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MECHANISM(S) OF FLUORIDE INDUCED CHLOROSIS [1, 2]

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Chlorosis and necrosis have long been recognized as the first visible symptoms of fluoride injury to plants (5, 6, 8, 10, 11, 12, 17). These symptoms occur when extraneous soluble fluoride compounds are introduced into the environment of either the roots or the leaves. In some cases, the lethal threshold concentration of tissue fluoride is reached suddenly, as the cells appear quite normal up to the time of visible injury. Solberg et al (16) and Adams and Solberg (1) described the microscopic injury, concurrent with the appearance of macroscopic symptoms, as a disintegration of chloroplasts followed by a collapse of the cells.

While visible symptoms have been studied extensively, only a few investigations have been made to determine whether or not any changes occur prior to the appearance of chlorosis and necrosis, or to describe the mechanism of action. Bandurski (2) found that in vivo synthesis of carotenes could be inhibited with fluorides. However, Bogorad (3) was unable to demonstrate any fluoride inhibition of the conversion of porphobilinogen to uroporphyrinogen or uroporphyrin, probable precursors to chlorophyll. Katz and Shore (7) suggested that fluorine compounds cause a degradation of chlorophyll by conversion to the corresponding pheophytins. The following investigation was initiated to elucidate further the mechanism of action of fluoride-induced chlorosis.

GENERAL EXPERIMENTAL PROCEDURES

Bush beans (*Phaseolus vulgaris* L. var. Burpee Tender Pod) or soy bean (*Glycine max* Merr. var. Bansei) were germinated and grown in vermiculite watered with 80 % Hoagland's solution. All treatments were made under controlled temperature at 24° C, and when necessary, exposed to constant fluorescent light at 400 ft.-c. Chlorophyll determinations were made according to the method of Koski (9), measuring chlorophyll a, chlorophyll b, and protochlorophyll at 663, 644, and 624 mμ.

Carotenes were separated by paper chromatography and measured at 446 mμ by the method described by Goodwin (4). Essentially, the procedure of Smith (14) was used to measure magnesium. A modification in this procedure consisted of dry ashing the chlorophyll samples. Absorption spectra were obtained using a Beckman DK2 ratio recording spectrophotometer.

FLUORIDE EFFECT ON PIGMENT SYNTHESIS. Bush beans were grown in the dark until the leaves were approximately one centimeter long. The stems were excised 2.5 cm below the cotyledonary node and placed in large petri dishes containing calcium-free, but otherwise complete, culture solution with and without fluoride (1.31×10^{-2} M NaF). The dishes were then exposed to light. At 24 hour intervals leaf samples were removed and the chlorophylls and protochlorophyll extracted and measured. The results (figs 1 & 2) are expressed in terms of milligrams pigment per ten plants. This was found to be as accurate for comparative purposes as dry weight measurements. The increase of both chlorophylls in the control plants followed a sigmoid curve (fig 1), but there was a definite reduction in the amount of both pigments when the plants were treated with fluoride. The effect of fluoride on protochlorophyll content is shown in figure 2. During the period of exposure to light the plants grew considerably, which would account for the apparent increase of protochlorophyll per plant in the control treatments. The plants exposed to fluorides did not show any increase in total protochlorophyll although they grew in a similar manner. These results suggest that fluoride may be inhibiting the synthesis of these pigments or causing their destruction.

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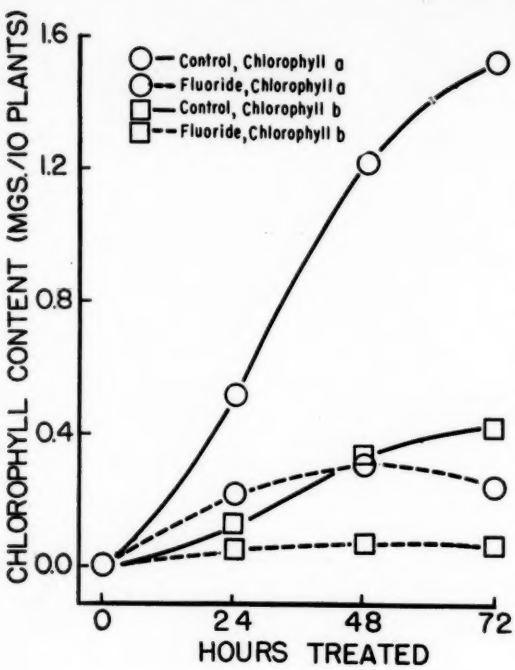

FIG. 1. The change in content of chlorophyll a and b in etiolated leaves treated with fluoride for 1, 2, and 3 days.

FLUORIDE EFFECT ON PROTOCHLOROPHYLL TO CHLOROPHYLL TRANSFORMATION. All procedures in this experiment were conducted in total darkness, with the exception of the 1 minute light exposure. Etiolated bush beans, 15 cm high, were excised at the base of the stem and partially immersed in culture solutions with and without fluoride (2.63×10^{-3} M NaF). After 61 hours treatment individual leaves were removed and placed in a spectrophotometer accessory kit as described by Smith, et al (15). This adapter is the same shape as the cell holder for the Beckman DK2 spectrophotometer, but holds a leaf between a piece of clear glass and a piece of opal glass. The opal glass window corrects for scattered light so that a consistent percentage is directed toward the photocell for each measurement.

The absorption spectra from 600 to 700 mμ were measured for the control and fluoride treated etiolated leaves. They were then exposed to 1 minute of light (incandescent, 1,000 ft-c) and the absorption spectra again measured.

Figure 3 shows a large decrease in protochlorophyll in both the treated and untreated leaves after exposure to 1 minute of light. At the same time there was an increase in the amount of chlorophyll present. After an additional 30 minutes in the dark there was very little change in protochlorophyll content, and the chlorophyll peak shifted only slightly. This shift has been described by Shibata (13). The changes in protochlorophyll and chlorophyll were almost identical in both the fluoride-treated and the untreated leaves. This indicates that fluoride does not affect the conversion of protochlorophyll to chlorophyll under the conditions of these experiments. If the reduction in chlorophyll content of fluoride treated leaves is due to an inhibition of chlorophyll synthesis, the inhibition must precede the protochlorophyll-chlorophyll transformation.

FLUORIDE EFFECT ON TOTAL ETHER SOLUBLE MAGNESIUM. Etiolated bush beans, 15 cm high, were excised 2.5 cm below the cotyledonary node and placed in large petri dishes with and without fluoride (5.26×10^{-3} M NaF). The dishes were then placed in a constant temperature chamber (24° C) at three different light intensities (60, 120, 400 ft-c). After 84 hours the leaves were removed and chlorophyll a and b, and total ether soluble magnesium were measured.

It is apparent from table I that the quantities of ether-soluble magnesium present under the different treatments varied in the same directions and with the same magnitudes as did the chlorophylls. If there was an inhibition of some step between an ether sol-

TABLE I

EFFECTS OF FLUORIDE (5.26×10^{-3} M NaF) ON CONCENTRATION OF CHLOROPHYLLS a & b, TOTAL CAROTENE, & TOTAL ETHER SOLUBLE MAGNESIUM COMPOUNDS IN EXCISED BEAN SHOOTS AT 3 LIGHT INTENSITIES

TREATMENT	LIGHT INTENSITY (ft-c)	CHLOROPHYLL a mg/g dry wt	CHLOROPHYLL b mg/g dry wt	ETHER SOLUBLE Mg, mg/g dry wt	TOTAL CAROTENES mg/g dry wt
Control	60	11.02	3.07	0.361	0.726
Fluoride	60	7.07	2.15	0.226	0.468
Control	120	11.24	3.26	0.361	0.684
Fluoride	120	8.26	2.42	0.328	0.516
Control	400	10.26	2.90	0.418	0.666
Fluoride	400	5.34	1.55	0.188	0.330
70 % Confidence limits		±0.48	±0.12	±0.043	±0.03

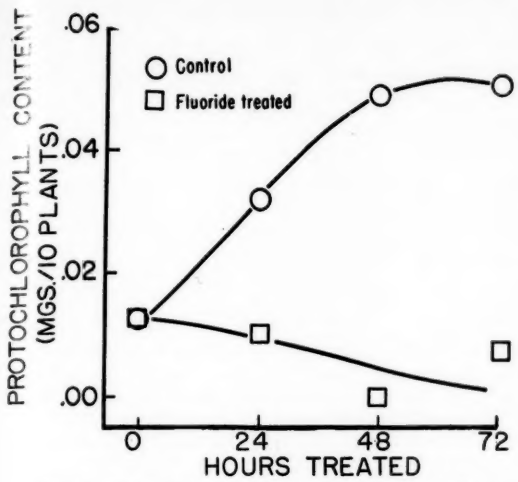

FIG. 2. The effect of fluoride on the content of protochlorophyll in bean leaves treated for 1, 2, and 3 days.

uble magnesium containing precursor and chlorophyll, one might expect an accumulation of this precursor. However, this was not apparent from these measurements. This suggests that if an inhibition of chlorophyll synthesis occurs, the inhibition must occur in the very early stages of pigment formation or even in the basic metabolism necessary for its synthesis.

FLUORIDE EFFECT ON CAROTENES. Young leaves from etiolated bush bean plants were removed and floated on calcium-free culture solutions in large petri dishes with and without fluoride (as NaF) at several concentrations. After 96 hours exposure to continuous light, the leaves cultured in low concentrations of fluoride were slightly chlorotic. The leaves were then removed, the pigments extracted, and the chlorophylls and carotenes measured (fig 4). The decrease in concentration of all three pigments was nearly linear with increase in fluoride concentration. When the decrease was calculated as per cent of control, it was essentially the same for all pigments. As a result, the rate of change of chlorophyll a with respect to the rate of change of total carotenes was a constant. These results show that fluoride reduces the concentration of the three pigments to a similar degree but do not indicate whether the reduction is due to a degradation of the pigments or to an inhibition of their synthesis.

FLUORIDE EFFECT ON CHLOROPLAST STRUCTURE. The leaves of fluoride-treated and control plants were sectioned and examined microscopically. As chlorosis developed, there was a simultaneous disintegration of the chloroplasts. This occurred prior to any apparent disruption of the cell. The breakdown of chloroplasts and simultaneous mixing of their contents with the cytoplasm could explain the results obtained in the previous experiments, that is, the decrease of all chloroplast pigments occurring simultaneously and to the same degree.

DISCUSSION & SUMMARY

Leaf tissue was cultured in a calcium-free mineral nutrient solution, with and without added fluoride, under constant light and temperature. Analyses were made on the chlorophylls, protochlorophyll, carotenes, and ether soluble magnesium compounds. The rate of change of the chlorophylls with respect to the rate of change of suspected chlorophyll precursors, and related compounds, was measured to investigate the mechanism of fluoride-induced chlorosis.

Sodium fluoride prevented the accumulation of chlorophyll a, chlorophyll b, and protochlorophyll in

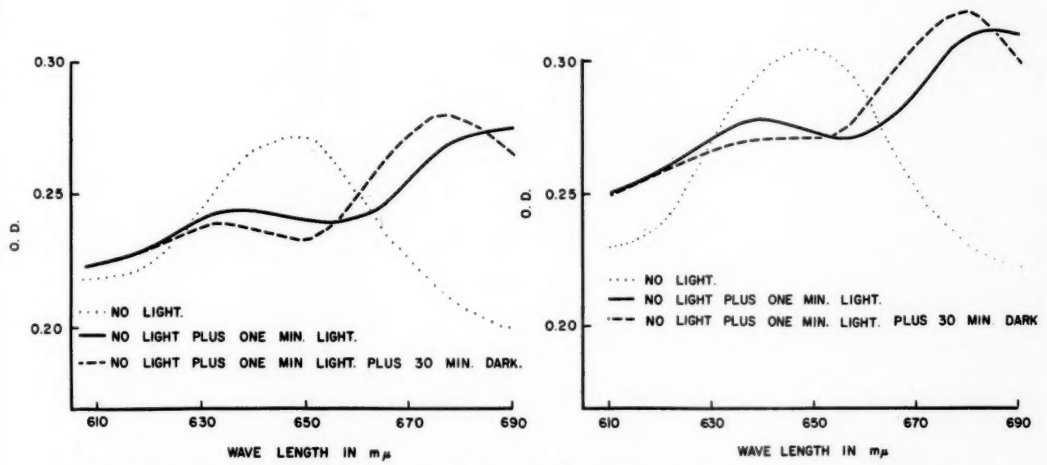

FIG. 3. The absorption spectra of etiolated leaves; fluoride treated (*left*) and untreated (*right*). The protochlorophyll peak is ca. 650 mμ and the chlorophyll peaks between 670 and 690 mμ.

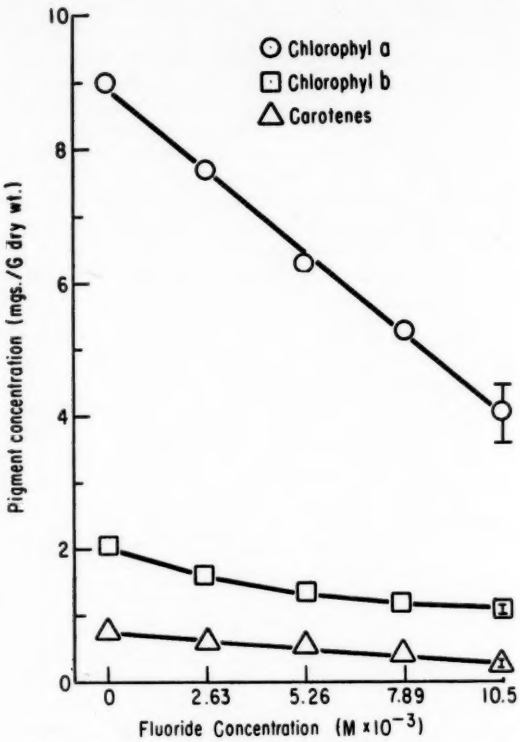

FIG. 4. The effect of fluoride concentration on chlorophylls a and b, and carotene concentration. Vertical bars indicate 70 % confidence limits.

bean leaves which were etiolated at the initiation of the fluoride treatment. However, the protochlorophyll to chlorophyll a transformation apparently was not affected. Chlorophyll a and b, the carotenes, and total ether soluble magnesium containing compounds were all affected proportionately. The similarity in magnitude of reduction of both the chlorophylls and total ether-soluble-magnesium containing compounds indicates there was no inhibition of pigment synthesis following the attachment of the magnesium atom to the ring structure.

These results suggest that if fluorides cause an inhibition of pigment synthesis, then this inhibition must occur very early in the synthesis of pigment components, or with some phase of basic metabolism necessary for their synthesis. The apparent dissolution of chloroplast structure that occurs concurrently with the appearance of chlorosis could explain the similarity in decrease of all pigments. Thus, fluorides may affect the early stages of pigment synthesis, or induce the degradation of chloroplast structure.

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FRUIT-INDUCED & APICAL SENESCENCE IN PISUM SATIVUM L.[1,2]

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Annual plants normally grow vegetatively for a limited time, flower, fruit, and then die. Rapid stem elongation of the Alaska pea plant continues until several fruit have begun development. During fruit development growth slows, and has stopped by the time fruit development is complete. Death of the vegetative parts soon follows (cf. 20).

It was Reichart (17) apparently, who first showed that flowering and fruiting are antagonistic to vegetative growth. He found that when flower buds were removed from the Vienna wall flower, normally an annual plant, this plant would continue vegetative growth for two or more years. When flowers were allowed to develop, the plant would rapidly set fruit and die. Numerous workers have substantiated and extended these results with other species (e.g. 6, 8, 9, 11, 12, 13, 19, etc.).

Various lines of evidence suggest that fruit may not act simply by diverting organic reserves from vegetative growth. Rather, the reproductive structures appear to cause death of the plants by some other mechanism.

Preliminary experiments indicated that periodic gibberellin treatments would substantially delay senescence in the Alaska pea. Further experiments were undertaken to study in greater detail the effect of gibberellin and fruiting on senescence of this plant. The evidence presented here indicates that the effect of gibberellin on senescence is indirect. Further experiments revealed a separate apical senescence in Pisum. The nature of this senescence is described.

Senescence will be considered here to mean those progressive, deleterious changes common to all organisms of a given species which ultimately lead to the death of the organism (18).

METHODS & MATERIALS

Plants of *Pisum sativum* L., var. Alaska, and in one experiment var. Alderman Improved (Ferry-Morse Seed Co., Los Angeles) were grown in the Earhart and Campbell Plant Research Laboratories as described previously (7).

Seeds were soaked in deionized water for 4 to 5 hours, then several seeds were sown in each of a number of 0.9 liter plastic containers in a 50-50 volume mixture of Vermiculite (expanded mica) and crushed rock. After germination for 4 days at 26° C in darkness, all except two vigorous seedlings were discarded from each pot. The two remaining seedlings were immediately moved to the appropriate greenhouse room and experimental treatments were begun. The plants were watered three times weekly with Hoagland's nutrient solution and with deionized water as required. The plants were grown throughout the year under solar radiation with the photoperiod extended to 16 hours by incandescent illumination. Substantial differences in growth rates were observed in the different experiments conducted during different seasons of the year. These effects can be ascribed principally to differences in solar radiation.

Growing temperatures are expressed as: day temperature (0800-1600 hr)/night temperature (1600-0800 hr). High growing temperatures (30°/24° C) were used when practical as a matter of convenience. With this temperature regimen, plants mature rapidly, are short, and easy to handle. The results indicate the same factor limits growth at high and at more moderate temperatures. Since plants grown at lower temperatures can be grafted much more successfully, plants for grafting experiments were always grown at 23°/17° C.

Gibberellin A_3 (Merck & Co., Rahway, N. J. & Eli Lilly Co., Indianapolis, Ind.) was applied where indicated as a single 4 μl ethanolic drop to the stem apex or to the youngest leaf. Three micrograms of gibberellin A_3 were applied weekly to each plant, beginning the day the plants were transferred to the greenhouse and terminating when growth had ceased. This gibberellin treatment is saturating so far as stem growth is concerned. Indole-3-acetic acid (IAA) and kinetin treatments were applied in a similar manner.

For sprays, solutions of 2 % sucrose, 1 % casein hydrolysate (Nutritional Biochemical Co., enzymatically hydrolyzed) and 1×10^{-3} M cobaltous chloride were made up in distilled water with 0.1 % Tween–20 (polyoxyethylene sorbitan monolaurate, a detergent produced by Atlas Powder Co.). Spraying was repeated twice weekly from germination until growth had ceased. Controls were sprayed with Tween–20 solution on the same schedule.

When root temperatures were to be different from top temperatures, seeds were planted directly in 1-gallon glazed crocks in which aluminum water coils had been placed previously. The seeds were germinated in the dark at 26° C and moved to the greenhouse rooms as usual. Thus, germination occurred under uniform conditions and temperature treatments were started only after the epicotyls had emerged. From the time the plants were moved to the greenhouses, water at the desired temperature was passed

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continuously through the coils. Control plants in this experiment were planted in identical crocks with coils in place but with no water circulation. Temperatures within the crocks were checked periodically with mercury thermometers which had been placed in the crocks at the time of planting.

Grafting was done by the method of Paton and Barber (16). Young (2-3 weeks old) plants were cut diagonally through the uppermost extended internode and the cut trimmed to a wedge shape. Older plants were cut directly through a node, since nodal tissue has been found to be more amenable to grafting in more mature plants (Highkin, unpublished). The sharpened scion was inserted into a verticle slit cut in the stock and held in place with a small rubber band. The grafts were protected from desiccation either with cotton, moistened periodically with water, or with wax of low melting temperature (50 % beewax-50 % paraffin oil). The relative success of cotton and wax-protected grafts varied. Overall success in grafting old plants ranged from 50 to 90 %. Side-grafts were made in essentially the same manner except that the stock stem was slit diagonally downward to about a third of the way through the stem.

When comparing the influence of red and far-red radiation the following sources were used. Red radiation was provided by filtering the radiation from six 8-foot pink fluorescent tubes through red cellulose acetate, and far-red by filtering the radiation from eight 40-watt Lumiline incandescent tubes through blue cellulose acetate. Red and far-red irradiation was given for 4 hours daily, immediately following the 8-hour daily light period. Intensity of the red source was approximately 300 μwatt · cm^{-2} and of the far-red 140 μwatt · cm^{-2} (in the far-red region), as measured with an Eldorado photometer equipped with a 1P29 RCA phototube (type S-3 surface). The photometer was calibrated at various wavelengths against the known output of the monochrometer of a Beckman DU spectrophotometer.

Height measurements were made weekly from germination until growth had ceased at maturity. When a plant was noticeably injured it was discarded and its previous measurements were expurgated from the data. Except where the influence of flowers and fruits was investigated all flowers were removed as soon as they became readily visible. Where statistical limits are indicated they express the standard deviation of the mean. Normally 12 plants were used per treatment.

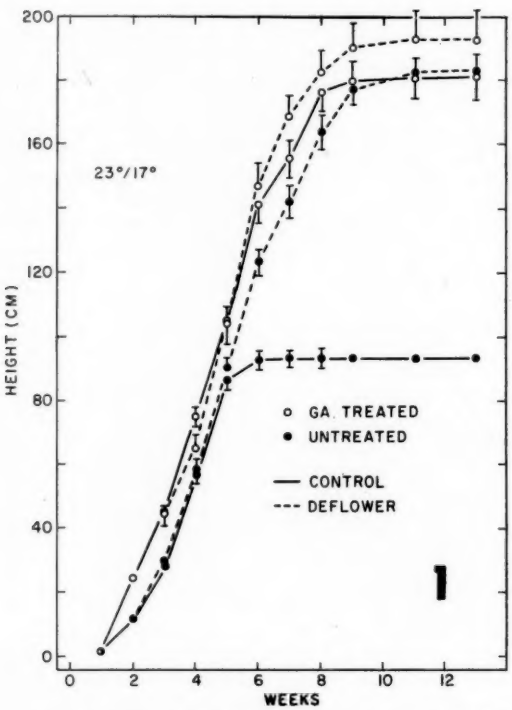


FIG. 1. Growth of Alaska peas in response to deflowering and gibberellin A_3 treatments. Control plants were allowed to develop normal fruit.

EXPERIMENTAL RESULTS

I. FRUIT-INDUCED SENESCENCE. *Effect of deflowering & gibberellin treatments on growth*: Since fruit development has already been shown to cause cessation of vegetative growth in other species, the influence of developing fruit on stem growth was investigated in the pea plant. Growth responses to periodic applications of gibberellin A_3 were also investigated. Results of a typical experiment are presented in figure 1. Removal of flowers as they formed did not influence growth rate but prolonged the period of active stem growth for several weeks. Gibberellin treatments caused an initial promotion of stem growth (due to prevention of light inhibition) but, generally, did not affect maximum growth rate, in agreement with previous reports (7). Applied gibberellin, however, prolonged the period of active vegetative growth nearly to the same extent as removal of flowers. Total vegetative growth of plants which received either of these treatments was almost as great as that of plants which were both deflowered and treated with gibberellin.

The rate of appearance of flowers was almost completely unaffected by gibberellin treatment. However, the first several flowers of gibberellin-treated plants shriveled and abscissed while on non-treated plants these first flowers set normal fruit.

Influence of the various treatments on final dry weight of mature plants is shown in table I. Deflowering resulted in a marked increase in final dry weight, as expected. Gibberellin treatments were found to cause a similar but smaller increase. These increases in dry weight are presumably due to the increased life span. A combination of deflowering and gibberellin treatments resulted in somewhat less total dry weight accumulation than deflowering alone. The reason for this decreased weight is not clear.

The average number of nodes and flowers formed per plant is included in table I. It may be seen that increased stem length was due primarily to an increase

TABLE I

EFFECT OF DEFLOWERING & OF APPLIED GIBBERELLIN ON ALASKA PEA PLANTS

TREATMENT	DRY WEIGHT PER PLANT (g)			AVG. NO. OF NODES	AVG. NO. OF FLOWERS
	VEGETATIVE PARTS	FRUIT	TOTAL TOPS		
Control	0.9 ±0.1	1.7 ± 0.2	2.6 ± 0.2	12 ± 0.5	2.8
GA-treated	2.2 ± 0.3	1.9 ± 0.2	4.2 ± 0.5	22 ± 1.2	12.6
Deflowered	5.9 ± 0.5	...	5.9 ± 0.5	24 ± 0.4	14.8
Deflowered & GA-treated	3.9 ± 0.4	...	3.9 ± 0.4	23 ± 0.9	10.9

Plants were grown at 23°/17° C. Details of treatments are described in the text. The plants were harvested when stem elongation had completely ceased.

in number of nodes formed. The increased number of nodes was the result of the prolonged growth period rather than an increase in rate of node formation. The substantially greater number of nodes formed in both the gibberellin and deflowering treatments accounts for the greater number of flowers formed as a result of these treatments. A flower is initiated at nearly every node formed after the first flowering node.

Gibberellin treatments were shown above to substitute for the effect of deflowering on stem growth. Since gibberellin treatments have been found here to markedly delay fruit-set it may be that the principal influence of gibberellin on prolonging stem growth is due to gibberellin-induced delay in fruit-set. In a separate experiment the mean fruit-bearing node was determined for gibberellin-treated and untreated plants. The average node bearing mature fruit on the control plants was 11.9 ± 0.2, while on the gibberellin-treated plants it was 17.2 ± 0.7. Thus, the bulk of the fruit was formed at a much later time on gibberellin-treated plants, supporting the suggestion that gibberellin delays senescence by delaying fruit-set.

Effect of developmental state of reproductive structures on growth: The presence of developing flowers and fruits results in an early termination of vegetative growth. The developmental stage of the reproductive structures at which they exert an effect on stem growth has also been studied. Flowers and young fruit were removed at several stages of development and the resultant effects on vegetative growth compared. The reproductive structures were removed: A, when the first white petals became visible in the flower bud (referred to as "first white petals"); B, when the flower petals had begun to dry (drying petals), or, C, when the young pods were 1 to 2 cm long. Control plants were allowed to develop mature fruit. The results are presented in table II. As in previous experiments, deflowering markedly prolonged stem growth, shown as an increase in final stem height. Removal of the reproductive structures resulted in prolonged stem growth of a similar magnitude regardless of whether the reproductive structures were removed when they reached the stage of young flowers, older flowers, or young fruit. These results indicate that the effect of the reproductive structures on vegetative growth is initiated by older fruit rather than through some action elicited by the flowers at synapsis or fertilization. This conclusion is consistent with that of Leopold et al (see Discussion). The slight increase in stem height found when flowers were allowed to remain until drying petals is not significant in this experiment. However, this trend may well bear further investigation.

Effect of seed development on growth: A further effect of applied gibberellin was found in an examination of the fruit formed on gibberellin-treated plants. While total fruit weight was not substantially decreased as a result of gibberellin treatment, the character of the fruit was markedly changed. In three

TABLE II

EFFECT OF REMOVING REPRODUCTIVE STRUCTURES AT DIFFERENT STAGES OF FLOWER & FRUIT DEVELOPMENT ON GROWTH OF ALASKA PEA PLANTS

DEVELOPMENT OF FLOWERS OR FRUIT WHEN REMOVED	DRY WEIGHT PER PLANT (g)			NO. OF NODES	HEIGHT AT MATURITY (cm)
	VEGETATIVE PARTS	FRUIT	TOTAL TOP WT		
Control (fruit developed to maturity)	0.4	0.6	1.0 ± 0.1	12 ± 0.4	62.3 ± 3.3
First white petals	1.9	...	1.9 ± 0.3	21 ± 0.5	101.0 ± 1.6
Drying petals	2.0	...	2.0 ± 0.3	22 ± 0.9	108.2 ± 6.9
Defruit (1–2 cm)	1.8	...	1.8 ± 0.3	22 ± 1.0	99.9 ± 7.1

Plants were grown at 30°/24° C. Control plants were allowed to form and develop fruit normally. In the other treatments reproductive structures were removed at various stages of development as indicated.

experiments seeds from fruit of untreated plants constituted an average of 57 % of total fruit weight, while on gibberellin-treated plants seeds constituted, on an average, only 14 % of the fruit weight. These results suggest that seed development rather than carpel growth might be specifically responsible for cessation of stem growth in the pea.

In order to test the influence of developing seeds directly, the fruits were allowed to develop normally, but the young seeds were cut loose from the carpel wall with a fine knife when the carpels had reached full size. Thus, the carpels expanded fully but seed development was arrested at an early stage. Results of a typical experiment are presented in table III. It is clear that prevention of seed development prolonged vegetative growth almost as long as did removal of flowers. It appears that the fruit causes senescence principally through an effect by the developing seeds. Plants whose fruit received comparable injury by inserting the knife blade through the carpel wall without detaching the seeds, behaved in a manner identical to that of the seeded controls.

Developing seeds are known to secrete auxin. In order to determine whether production of auxin by the seeds was partly or wholly responsible for the influence of the seeds on vegetative growth, IAA was applied periodically to deseeded fruit. Four micrograms of IAA (in ethanol) were applied to each deseeded fruit twice weekly until two subsequent fruits had developed on the plant. The treatments were continued on newly developing fruit until growth ceased. No effect of the IAA treatment could be observed. Deseeding prolonged vegetative growth to the same extent whether IAA was applied or not.

II. Apical Senescence. Deflowering and gibberellin treatments resulted in prolonged vegetative growth. However, these treatments were effective for only a limited time (or for only a limited amount of growth). Stem growth ultimately ceased regardless of any experimental treatment so far tested. In order to identify the factor responsible for the ultimate cessation of stem growth, experiments were undertaken to determine what portion of the plant first ceases its normal function.

Morphology of apex when stem growth ceases: Termination of stem growth, in the absence of fruit, occurred with the formation of a final node consisting of a typical bract and leaf, and a single flower. No indication of the stem apex remained (fig 2). The obvious conclusion is that this is a terminal flower and we are dealing simply with determinate growth. However, this is not the case in a physiological sense. The total number of nodes (& number of flowering nodes) formed before growth terminates may be readily changed by temperature (table IV), and light treatments (table VI). Thus, the factor which determines whether stem growth shall continue or whether the remaining apical meristematic tissue shall be invested entirely in a single flower is the factor which we are investigating. An important fact verified by figure 2 is that the upper portion of the plant was still green and apparently completely healthy at the time stem elongation ceased. In deflowered plants the leaves usually turned brown and died 1 to 2 weeks or more after stem elongation had ceased.

Effect of root & shoot temperatures on growth: Growing the aerial portions and roots of the plant at different temperatures could provide evidence indicating which of these plant parts first ceases normal functions, and thus causes growth to cease. At 30°/24° C stem growth is rapid but the plant lives for only a relatively short time. At lower temperatures (e.g., 17°/11° C) growth is somewhat slower but continues for about 2-3 times as long and final stem height is much greater (20, 7). In the following experiment the aerial portions of certain plants were grown at either 30°/24° C or 17°/11° C while the roots were maintained at either 30° or 17° C (without day-night fluctuations). Flowers were removed regularly from all plants. Rates of stem growth in such

Table III

Influence of Developing Seeds on Vegetative Growth of Alaska Peas

	Control	Deseeded	Deflowered
Final stem height (cm)	68.1 ± 1.7	105.8 ± 3.7	124.4 ± 5.4
No. nodes/plant	12.9	24.1	26.5
" laterals/plant*	0	12.3	17.4
" flowers/plant	4.8	14.0	15.0
" pods/plant	1.4	5.5	...
Dry wt (g/plant)			
Vegetative parts	0.35	1.42	2.69
Pods	0.62	0.99	...
Seeds	0.49	...	...
Total fruit	1.11	0.99	...

Plants were grown at 26°/20° C. On control plants the fruit was allowed to develop normally, on deseeded plants the young seeds were detached from the carpel wall at the time the fruit had reached full size. Measurements were made after all plants had died.

* Laterals were removed as soon as possible after growth began.

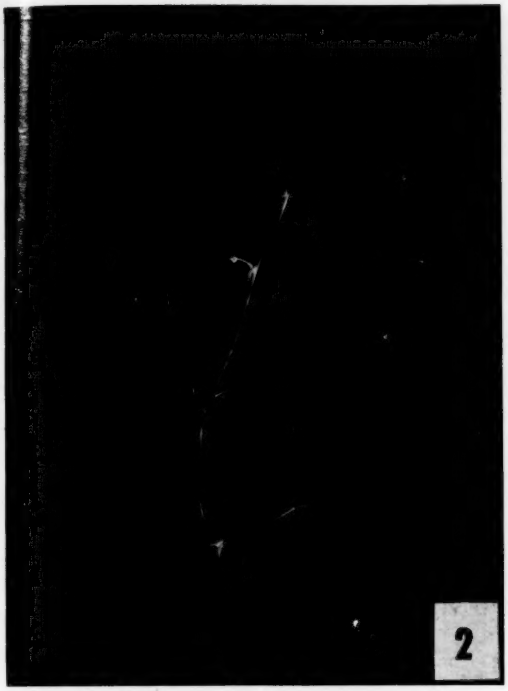


FIG. 2. Appearance of the stem apex of a deflowered Alaska pea plant shortly after differentiation of the final node. The nearest bract has been removed to show the final flower and the absence of an apical meristem. Earlier flowers were removed as they formed.

an experiment are presented in figure 3. It is obvious that rate and extent of stem elongation were determined almost entirely by the temperature of aerial portions of the plant. The influence of root and top temperatures on weight of roots and tops and on node number is shown in table IV. It may be seen that root temperatures had a substantially greater influence on dry weight than on stem elongation. Roots, then, do not limit stem elongation under the conditions used here.

This experiment also demonstrates the influence of two growing temperatures on duration of stem growth and number of nodes formed prior to senescence. At high temperatures growth had ceased and the plants were dead at harvest. At lower temperatures more nodes had been formed per plant at the time of harvest and the plants at low temperatures were still growing vigorously. It must be expected that the plants grown at 17° C would have continued growth for a substantially greater time and formed many more nodes prior to senescence if growth had been allowed to continue. The visual appearance of the stem apex makes it possible to anticipate senescence some time in advance of growth cessation. No indications of future senescence could be observed in these plants at the time of harvest.

Effect of sucrose & other chemical treatments on growth: In an effort to learn whether cessation of stem growth is related to a deficiency occurring in the photosynthetic apparatus or in nitrogen nutrition, deflowered plants growing at 30°/24° C were sprayed twice weekly with either 2 % sucrose or 1 % casein hydrolysate solution. Plants sprayed with 0.1 % Tween–20 solution served as controls. Sucrose and amino acids had almost no effect on growth, and neither affected time of senescence. The negative results here make it impossible to determine whether sucrose and amino acids were not limiting for growth or whether they did not enter the plants in sufficient amounts to influence growth. Ascorbic acid sprays also were found to be without growth-promoting effects under these conditions (*2*). Indoleacetic acid, applied weekly as ethanolic drops at doses of 4.0, 0.04, or 0.0004 μg per plant, was without effect on either growth rate or time of senescence, except that the highest dose resulted in a slight growth inhibition.

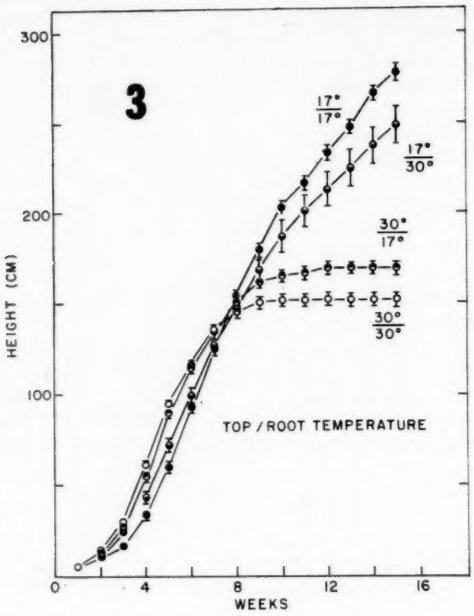
In further experiments at a growing temperature of 26°/20° C, 2 % sucrose was sprayed twice weekly onto plants which were allowed to set and develop fruit normally. Sucrose sprays were completely ineffective also in delaying fruit-induced senescence. Semi-weekly sprays with 1×10^{-3} M cobaltous chloride substantially delayed decoloration and death of the leaves, but had no effect on growth or time of either fruit-induced or apical senescence. Weekly

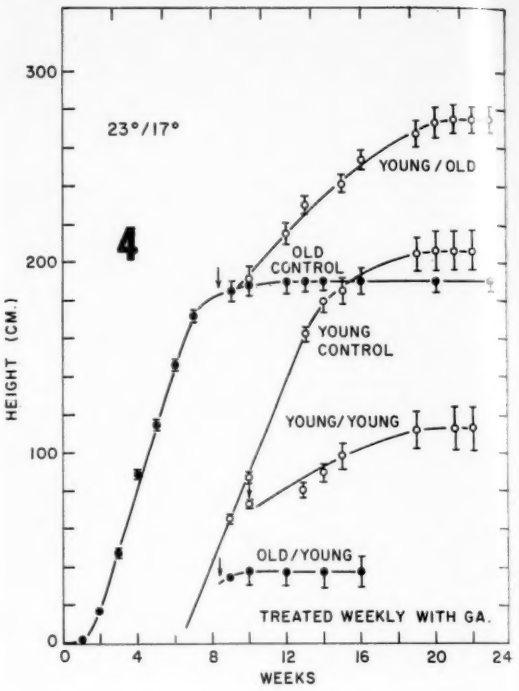
TABLE IV

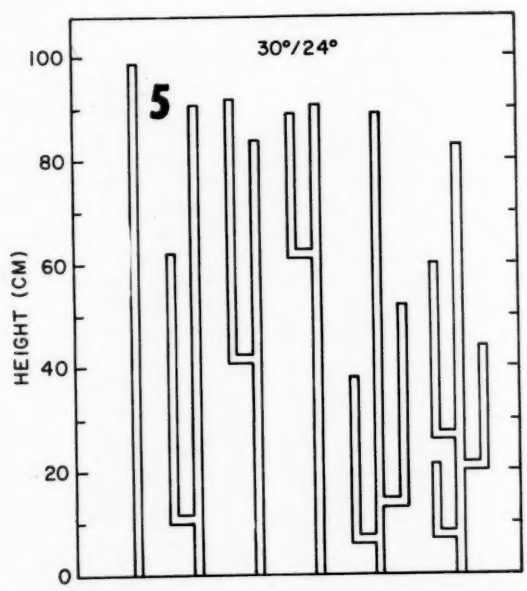
EFFECT OF ROOT & SHOOT TEMPERATURE ON GROWTH OF ALASKA PEAS AFTER 15 WEEKS GROWTH

TEMP. (C) (TOP-ROOT)	STEM HT. (cm)	AVG. DRY WT/PLANT (g)		NO. NODES
		TOP WT	ROOT WT	
17°/17°	277 ± 5	34.8 ± 2.7	9.2	34 ± 0.6
17°/30°	250 ± 10	21.3 ± 2.0	1.6	34 ± 0.8
30°/17°	172 ± 2	8.7 ± 0.4	1.7	32 ± 2.0
30°/30°	153 ± 3	6.2 ± 0.4	0.9	27 ± 0.8

Final measurements were made after 15 weeks, when plants with tops at 30° C had ceased growth, while those at 17° C were still growing vigorously. All plants were treated weekly with gibberellin A_3 and deflowered periodically.







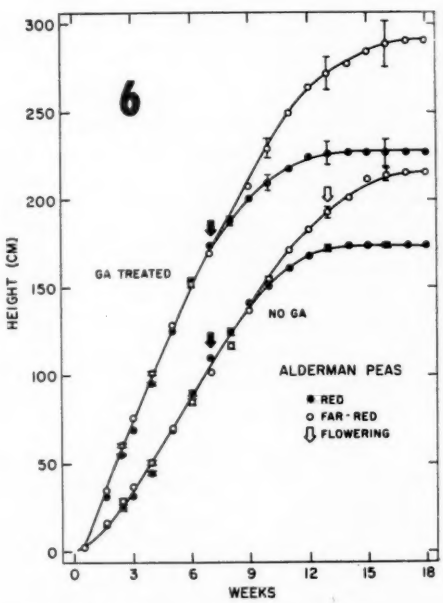


FIG. 3. The influence of root and top temperatures on stem growth of Alaska pea. Plants grown with tops at 17° C were still growing vigorously at the time of harvest while those whose top temperature was maintained at 30° C, were mature.

FIG. 4. Growth of Alaska peas illustrating the growth of young or old stem tips grafted onto young or old plants. The labels indicate scion/stock.

TABLE V

INFLUENCE OF SIDE-GRAFTED STEM TIPS ON STEM GROWTH & SENESCENCE

STOCK	ORIGINAL APEX	AGE OF GRAFTED APEX	HEIGHT OF STOCK AT TIME OF GRAFTING	GROWTH OF STOCK STEM AFTER GRAFTING	GROWTH OF GRAFTED SHOOT
Old plants	Control		112 cm*	17 ± 4.3 cm	
	Removed	Young	107		42 ± 4.7 cm
	Present	Young	101	16 ± 3.4	18 ± 6.5
Young plants	Control		36*	159 ± 18.0	
	Removed	Young	21		12 ± 2.0
	Removed	Old	23		1 ± 0.5
	Present	Young	17	113 ± 25	20 ± 9.0
	Present	Old	25	117 ± 8.4	2 ± 0.5

Plants were grown at 23°/17° C under 16 hour photoperiod. All plants were regularly deflowered.
* For the ungrafted controls, height at the time grafts were made is shown.

treatments with ethanolic solutions of kinetin at 100 and 1,000 ppm were also without effect.

Grafting experiments: A number of grafting experiments were conducted to determine if the stem apex itself is primarily responsible for cessation of apical growth. Stem tips from young plants were grafted to the tops of older stems and, reciprocally, the stem tips from the older plants were grafted to seedling plants. Results of several such experiments have proven quite conclusive.

In a typical experiment, stem tips were cut from nearly mature plants (i.e., plants which could be expected to cease stem growth within 2-3 weeks). Young (2-week-old) stem apices were grafted to the tops of the old stems in their places. The young stem tips resumed growth after 10 to 12 days and grew at a moderately rapid rate (fig 4). They continued growth for 8 to 10 weeks beyond the time the old plants normally matured. It may be seen from the figure that young tips grafted to old plants grew as fast and for as long a time as young tips grafted back to young plants. While growth rate of grafted tips was less than that of young intact plants, the grafted tips continued growth for fully as long as did the intact plants.

Tips from old plants may be grafted to young seedlings with good success, judged by the strength of tissue union and the fact that grafted tips, with several leaves present, remain completely turgid and green for several weeks. However, no more than 1 to 2 cm growth occurred in these plants. Allowing 1 to 2 weeks for tissue union to occur, these old tips grew only as much as stem tips left intact on the old plant. These grafting experiments have been repeated with plants treated periodically with gibberellin and with untreated plants. The results have always been substantially the same as reported above.

The old plant, then, was fully capable of supporting stem growth for a much longer time and to a considerably greater total height than normally occurs. However, the old stem apex did not continue growth even under what would appear to be optimum conditions, i.e., when grafted onto a young, vigorously growing plant. Thus, cessation of growth of the pea stem in the absence of developing fruit is due to a decline in vigor of the growing point or immediately subjacent tissue.

Non-transmission of controlling factor: Further grafting experiments were undertaken to test for transmission of a possible juvenile or senescence factor. Nearly mature stem apices were side-grafted to stems of young plants about two to four centimeters below the tip, leaving the stem tip of the stock intact (table V). The old tip grafted to a young seedling had virtually no effect on growth of the seedling. Furthermore, young tips side-grafted near the apex of old plants had no effect on maturation of the old stem tips. Growth of young, grafted tips was initially inhibited by apical dominance of the old tips. As soon as the old tips ceased growth, the young side-grafted shoots resumed normal growth. In both cases, then, presence of a stem tip of a different age had no effect on growth of the intact plant. Thus, neither an aging factor nor a juvenile factor capable of being translocated across a graft union seems to be responsible for apical senescence in this plant.

Lateral stem growth: On plants which had been deflowered, lateral buds began growth shortly before

FIG. 5. Final length of main stem and laterals allowed to develop on deflowered Alaska peas. Both length of laterals and position of insertion on the main stem are to scale.

FIG. 6. Growth of Alderman Improved peas in response to gibberellin A_3 treatment and daily red or far-red irradiation. The arrows indicate the time of appearance of the first visible flowers. No flowers ever became visible on the gibberellin-treated, far-red irradiated plants. All flowers were removed as soon as they became visible.

growth of the main stem ceased. (No laterals grew out when fruit was allowed to develop.) Normally, in the experiments described here, these laterals were removed as soon as they began growth. In a separate experiment growth of selected laterals was followed to provide further information on the nature of apical senescence. After the laterals began to grow, plants were trimmed to the main stem and a single lateral branch originating near the bottom, middle, or top of the main axis. Other plants were allowed to develop either two or three laterals from near the base of the old stem. No other laterals were permitted to develop. The laterals continued growth for several weeks. Duration of growth of laterals was approximately equal to the duration of growth of the main stem, although growth rate was not as rapid as had been that of the main axis. Final height of the main stem and laterals is illustrated diagrammatically in figure 5. The average position of insertion of lateral shoots on the main stem, as well as their final length is represented to scale. Total growth of the laterals was about equal, whether the shoot originated near the base or middle of the old stem. Lateral shoots from near the top of the old stem grew less. While total growth of upper laterals was least, their rate of growth was the most rapid. Thus, they could hardly have been deficient in water or nutrients as a result of difficulties of translocation up the long stem. When two or three shoots were allowed to develop on a single plant, each shoot grew less than if only one lateral was allowed to develop. Apparently in this case competition for nutrients or water occurred.

Growth of lateral shoots, after the stem apex had ceased growth, confirmed the conclusions reached as a result of the grafting experiments. Cessation of growth of the stem apex is the result of some effect localized in the stem tip itself. Furthermore, growth of laterals indicates that meristematic cells within the old plant are capable of continued growth. Thus, not all the meristematic tissue of the stem becomes senescent at the same time.

III. Flowering in Relation to Apex Senescence. Certain biennial plants, and plants whose flowering is strictly controlled by photoperiod, will grow far longer than their usual life cycle when kept strictly vegetative (e.g. 3, 4, 5). In many of the cases studied, growth appeared to be capable of continuing indefinitely. The initiation of flowers, even though all flowers are removed as soon as they are formed, may have some physiological effect on the stem apex, causing the gradual loss of some essential capacity. Experiments described below indicate that the onset of flowering does not initiate or substantially promote apical senescence in Pisum.

Alaska and Alderman Improved varieties of pea were grown in 8 hours natural light per day, supplemented with either red (low intensity red fluorescent) or far-red (blue & far-red radiation from incandescent bulbs) radiation for 4 hours at the beginning of the daily dark period. Day temperature was 26° C and night temperature 23° C. All flowers were removed as they formed, and gibberellin treatments were included in each light condition. (Aqueous solutions of gibberellin were used on the Alderman peas since previously it had been found that ethanol, over a period of time, caused considerable injury to this variety.)

Results for the Alderman variety are presented in figure 6. The far-red treatment alone did not completely prevent flowering in this variety, but it did delay the onset of flowering for several weeks. The average first node to flower in far-red treated plants was 30.2 compared to 22.4 for red-treated plants. Weekly treatments with gibberellin had no effect on flowering time of red-treated plants, but completely prevented flowering of far-red-treated plants. In spite of the marked delay in flowering, senescence was only slightly delayed by far-red as compared to red treatment and took place regardless of whether or not flowering occurred.

An identical experiment with Alaska peas gave generally similar results (table VI). With Alaska peas, surprisingly, no flowering occurred on far-red irradiated plants regardless of whether or not they received gibberellin treatments. Senescence occurred at nearly the same time in all treatments, whether the plants had begun flowering or not. Neither red nor far-red irradiation had any effect on growth rate (tall peas are, apparently, virtually saturated with respect to endogenous gibberellin), but red irradiation resulted in a substantial increase in rate of node formation compared to far-red treatment. Plants irradiated

Table VI

Influence of Red/Far-Red Radiation & Gibberellin Treatments on Growth, Flowering, & Senescence of Alaska Pea

Treatment	Height at senescence (cm)	No. nodes at senescence	Age at senescence	1st Flowering node
Red	71.5 ± 3.4	22.6 ± 0.8	ca. 70 days	19.4 ± 0.7
Red + Ga	123.4 ± 2.2	22.0 ± 0.5	"	17.1 ± 0.3
Far-red	84.6 ± 3.3	17.4 ± 0.7	"	No flowers
Far-red + Ga	109.4 ± 3.4	15.6 ± 0.7	"	No flowers

Plants were grown at 26°/23° C under 8 hours solar radiation followed by 4 hours red or far-red radiation daily. All flowers were removed as they formed. Gibberellin was applied weekly to indicated treatments.

with far-red generally ceased growth before forming those nodes which, in red-treated plants, were the first to initiate flowers. No examination was made for the presence of microscopic flower primordia. However, the fact that in the Alaska variety senescence occurred in the far-red-irradiated plants before formation of what would have been the earliest flowering nodes indicates that no flower primordia were present in this variety, at least. Thus, apex senescence appears to be independent of flower initiation in this species.

Discussion

Two separate factors can result in termination of stem growth in the Alaska pea. The first factor, which may be expected to limit growth during normal plant development, is a result of some action of the developing fruit. As a result of fruit development the entire plant dies, with the exception of the seeds. Two general mechanisms can be envisaged: fruits may divert nutrient or hormonal materials from the rest of the plant, thus terminating stem growth; alternatively, fruits may secrete some factor which inhibits stem growth.

Leopold et al (6) have reported that the longer fruit is allowed to develop before removal, the greater its effect on leaf senescence; but they state that even when fruit is allowed to attain full size, its removal delays senescence. Our results suggest that young fruits have a relatively small influence on senescence in the pea as measured by cessation of stem growth. Removal of young fruit is nearly as effective as removal of flowers in delaying senescence. The greatest inhibitory effect appears to be exerted by developing seeds. When seed development was prevented surgically, stem growth was markedly prolonged, continuing nearly as long as that of deflowered plants. The seeds alone do not completely control vegetative growth, since in a number of species parthenocarpic fruit will also retard growth (e.g. 15). The effect of parthenocarpic fruit and deseeded fruit may well be due to diversion of major nutritional factors. However, the apparently specific influence of the seeds tends to support the conclusions of many workers that diversion of organic reserves cannot account completely for the effect of developing fruit on senescence. Leopold et al (loc. cit.) have shown that staminate plants of Spinacia die following maturation of the reproductive structures, just as pistillate plants die following fruit maturation. Pollen development could hardly be expected to divert sufficient reserves from the rest of the plant to cause death by starvation.

Periodic gibberellin treatments delay senescence in the pea by delaying fruit-set and inhibiting seed development. Whether or not gibberellin has any additional effect on fruit-induced senescence cannot be determined, since the effects of developing fruit and gibberellin are both terminated by apical senescence. Thus, whether or not their effects are additive cannot be established. It is clear that gibberellin has no effect on apical senescence.

Even when flowers are continually removed from the Alaska pea plant, or gibberellin applied, or both, growth of the main stem is extended only for a limited time. Under these conditions senescence of the stem apex is evidently determined by a second factor. It has been demonstrated here that cessation of growth of the stem apex results from some degenerative change localized within the apex itself. Neither common nutritional factors (sucrose, amino acids), nor the temperature condition under which the roots grow, significantly affect the onset of apical senescence. (Apical senescence is markedly delayed by lowering the growing temperature of the tops.) The roots and stem have been shown to be capable of supporting growth for at least several weeks beyond the onset of apical senescence. It is also apparent that whatever factor controls apical senescence is not readily translocated within the plant. Thus, neither a translocatable juvenile factor nor a senescence factor appears to be involved here. The important point here is not that some growth factor normally supplied by another part of the plant is missing from the apex, but that the capacity of the apex to produce some factor essential for its own continued growth has been lost. For example, the possibility that some nutrient factor becomes exhausted has been eliminated by the present experiments. Such a nutrient factor would be restored to old stem apices grafted to young plants.

Developing fruits have a systemic effect, completely inhibiting growth of all lateral buds as well as the main axis. Apical senescence, however, is localized to the degenerate tip, since other tissue in the stem is still capable of vigorous growth. Lateral buds arise from the apical meristem some time prior to senescence, when it is younger and has undergone fewer mitotic divisions. Perhaps the greater growth of lower lateral branches compared to laterals originating from the upper portion of the plant is a reflection of the origin of lower buds from younger meristems, that is, from meristematic cells which have previously undergone fewer total cell divisions. Degeneration of vegetative cells as a result of continued cell multiplication has been observed in various organisms (Lemna, 1; fungi, 10, & protozoa 14).

Many plants when kept strictly vegetative (for example, by appropriate photoperiodic treatment) apparently are able to continue growth far longer than comparable plants which form flowers, even if no fruits are allowed to develop on the flowering plants. This has been directly demonstrated only with spinach, but in this case a complete change in growth habit is associated with floral induction. In other cases, no flowering-deflowered control has been included. It has been possible to keep Alaska and Alderman Improved peas vegetative (non-flowering) by certain light and gibberellin treatments. Even though these plants remain vegetative, apical senescence occurs at almost the same time as in flowering (but deflowered) plants. Senescence of the apex, then, may occur even in the absence of the flowering condition.

The senescence problem studied here is distinct from the problem of leaf senescence studied by various workers. Leaf senescence occurs also in the Alaska pea, progressing from base to tip. However, all our observations indicate that leaf senescence is an independent phenomenon, with no direct relationship to the apical senescence studied here. As pointed out earlier, apical senescence is often complete while most of the leaves are still green and healthy. When the developing fruits cause senescence the leaves die at the same time as the stem apex. Developing fruit thus may have the effect of markedly hastening senescence of the leaves.

It is not known if the phenomenon of apical senescence is widespread among higher plants. Certainly many species do not seem to show comparable behavior. On the other hand, there are other species in which one or more lateral buds assume dominance after the main stem has made the early growth. In rosette plants, too, lateral tillers often become more vigorous than the primary apex. These cases may prove to be caused by a degeneration of the stem apex, comparable to the case of Pisum, reported here.

Summary

Alaska peas, like many other annual plants, normally die as a result of fruit development. Gibberellin treatments delayed senescence by delaying fruit formation. Detachment of the seeds was almost as effective as removing the entire fruit in prolonging vegetative growth. When fruit development was prevented by deflowering or by gibberellin treatments, growth of the main stem continued only for a limited time. Ultimately the stem apex differentiated into a final flower and leaf and the apical meristem disappeared.

The stem apices of young plants, grafted to the tips of old plants, grew as rapidly and for as long a time as when grafted back to a young plant. The old tips ceased growth even when grafted to a young plant. An old or young stem apex side-grafted to another plant did not substantially affect growth of the plant to which it was grafted.

When fruit was allowed to mature on the Alaska pea the entire plant died. However, when flowers were continually removed, and apical senescence occurred, numerous laterals began growth. When individual lateral branches were allowed to develop, their growth rate was less than that of the old stem but duration of growth was substantially as long. It is concluded that apical senescence is due to some degenerative change taking place within the apex itself and is not translocated within the plant.

Apparently, senescence of the apex is not directly related to the physiological onset of flowering. Apical senescence occurred even though no flowering took place.

Acknowledgments

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PHOTOSYNTHESIS & TRANSPIRATION FROM UPPER & LOWER SURFACES OF INTACT BANANA LEAVES.[1]

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The use of modern gas analysis instrumentation makes it possible to obtain continuous recordings of transpiration (3, 6, 8, 12, 13, 23) and photosynthesis (4, 5, 16, 19) from intact portions of higher plants. The high sensitivity and rapid response of these instruments permits one to determine rather subtle effects of various internal or environmental factors on physiological processes involving gas exchanges between the plant and its environment (4, 7, 8, 13).

Measurements of photosynthesis and transpiration comparing the rates on the two surfaces of leaves have been made in the past (9, 10, 17, 18, 24), but none of these reports is of very recent origin. In general it was found by these investigators that the rates of photosynthesis and transpiration were dependent upon the stomatal distribution on the two leaf surfaces. Conflicting conclusions have been reached, however, concerning the extent to which these gas exchange processes are limited by the degree of aggregate stomatal opening. Lloyd (14) has concluded that the opening and closing of stomates should be without appreciable effect on the transpiration rate. Conversely, Thoday (22) and Maskell (15) consider that changes in stomatal aperture definitely influence the rate of photosynthesis. Freeland (9) concluded that in some plants, especially those with a thick cuticle, the stomates constitute the only path of entry of CO_2 into the leaf; whereas in other plants considerable quantities of CO_2 could enter the leaf directly through the epidermis.

More recently, Andersson and Hertz (3) have interpreted the periodical oscillations in the transpiration curve of Thunbergia as being due to a periodical opening and closing of the stomates. Andersson and Hertz (3), as well as Allerup (1) and Decker and Wien (8), have observed a temporary stimulation of transpiration from a leaf following its excision from the parent plant. Allerup (2) considers this increase in transpiration rate to be due to an increase in stomatal opening which he has demonstrated to take place following such treatment.

The work here reported was undertaken preliminary to an investigation of the effects of various spray materials on the processes of photosynthesis and transpiration in banana leaves. For this purpose it became necessary to devise appropriate facilities to determine the relationship between these two processes and the leaf surface from which they were measured.

MATERIALS & METHODS

The plants used in this study were young potted specimens of *Musa acuminata* L. var. Hort. Gros Michel. The plants were obtained as small rhizome pieces (so-called button seeds) from Costa Rica and planted in 9 inch pots in the greenhouse. At the time when the plants were used, they were 8 to 9 months old and about three feet tall (from soil line to leaf crotch).

Photosynthetic rates were determined with an infrared CO_2 analyzer (Beckman's model 15 A) connected to a strip chart recorder (Texas Instrument Rectiriter 1 milliampere recorder). With this instrument the CO_2 content of an air stream was measured before and after it passed over a given area of leaf surface enclosed in a plexiglass leaf chamber as described below.

Transpiration rates were determined by measuring the absolute humidity of an air stream before and after it flowed over a known area of leaf surface. The humidity measurements were done on a Dynalog Dew Point Recorder (Foxboro Co.). This instrument consists of a humidity sensitive element (a Dewcel), a power source, and a strip chart recorder. This instrument was factory calibrated to record the humidity of the surrounding air in terms of its dew point temperature ° F.

The leaf surface, upon which the measurements were conducted, was enclosed in a plexiglass leaf chamber. Two different leaf chamber designs were used. The first type of chamber (fig 1) enclosed a leaf flap, prepared by making two parallel tears, 10 cm apart, in the blade from the margin to the midrib. The leaf flap was inserted through a narrow slit into the inner portion of the leaf chamber, and the slit sealed with modeling clay. The air stream entered the inner portion of the chamber through a manifold

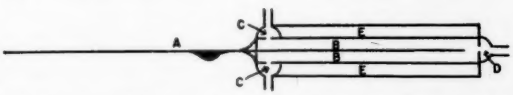

FIG. 1. Diagram of first type of leaf chamber enclosing a leaf flap. A. Banana leaf. B. Air chamber. C. Air intake manifold. D. Air exhaust manifold. E. Water jackets. Water intake and exhaust manifolds are not shown.

[1] Received November 14, 1960.

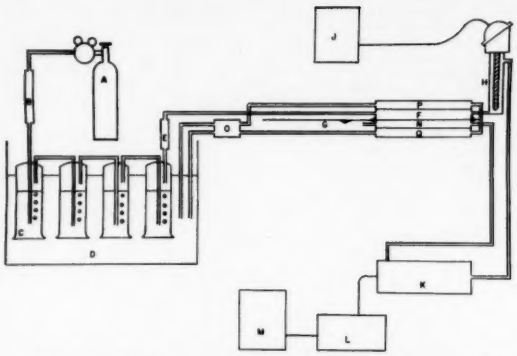

FIG. 2. Diagram of the arrangement of the apparatus. A. Bottle of compressed air. B. Flowmeter. C. Sulphuric acid bubbler. D. Constant temperature water bath. E. Cotton filter. F. Air chamber on upper leaf surface. G. Banana leaf. H. Humidity sensitive dewcell. J. Strip chart recorder. K. Infrared CO_2 analyzer. L. Amplifier. M. Strip chart recorder. N. Air chamber on lower leaf surface. O. Circulating water pump. P. and Q. Water jackets.

which divided it equally over the two leaf surfaces and thence to a single exhaust manifold in the opposite end of the chamber. Water from a constant temperature bath (30° C ± 0.5° C) was circulated through a plexiglass water jacket surrounding the leaf chamber.

The second type of leaf chamber (fig 2) consisted of two box-shaped halves attached with modeling clay to opposite leaf surfaces. Each half consisted of an air chamber, with appropriate inlet and outlet manifolds, and a unilateral water jacket with an inlet and outlet. The air chamber was bound on its outer surface by the water jacket and on its inner surface by the leaf surface to which it was attached. The two box-shaped chambers were connected in series so that by reversing the direction of the air stream through them and by inserting the Dewcel element and the sample tube of the CO_2 analyzer before, between, or after the chambers, it was possible to measure the humidity and CO_2 content of the incoming air, or of the air after it had passed over one or both leaf surfaces. The second type of chamber had three advantages over the first type: A, it permitted separate measurements on the two leaf surfaces, B, it eliminated the inclusion of the two torn edges of the leaf flap in the chamber, and C, it always enclosed the same amount of leaf surface area (10 × 10 cm).

The CO_2 content of the incoming air stream was maintained at 300 ppm by using a tank of compressed air as the source of air. The humidity of the incoming air stream was maintained at a dew point of 59° F (at 30° C this corresponds to a relative humidity of 40 %), by passing it through a series of four bubbling towers containing sulphuric acid of a specific gravity of 1.384. The bubblers were maintained at 30° ± 0.5° C in a constant temperature bath. A small pump circulated water from the constant temperature bath to the water jackets on the leaf chambers.

Figure 2 shows, diagrammatically, the arrangement of the apparatus used. The rate of air flow was adjusted by a needle valve on the compressed air bottle and determined by a flow meter inserted in the air stream before it passed through the sulphuric acid bubblers. A bank of eight 8-foot fluorescent lights (General Electric Power Grove lamps) was mounted above the plant, with the leaf chamber in place on one of its leaves. The bank of lights could be raised and lowered by a pulley and crank arrangement. At a distance of 6 inches from the lamps, the light intensity, as measured by a Weston illumination meter, was 3,000 ft-c. Very low light intensities were obtained by raising the lights to their highest position and by placing different thicknesses of white tissue paper over the leaf chamber.

Throughout the experiments a standard procedure was used (fig 3 C) which consisted of exposing the plant, with a leaf chamber attached to one of its leaves, to the following sequence of events: A, 15 minute period of darkness during which the CO_2 content and dew point temperature of the incoming air was recorded; B, a 15 minute period of darkness during which the CO_2 content and dew point tempera-

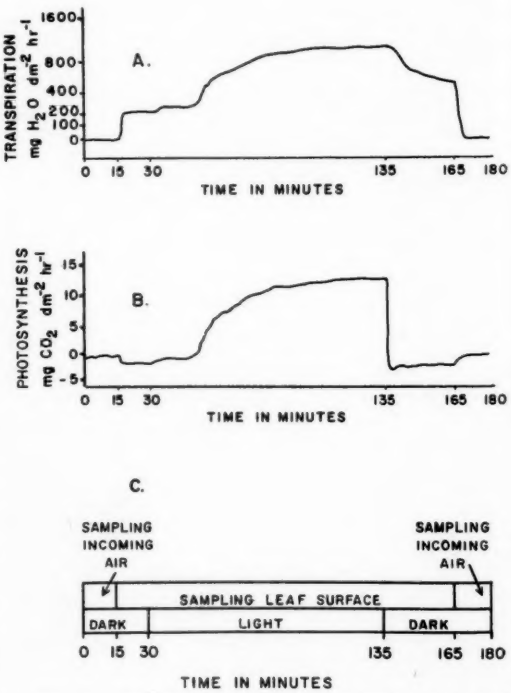

FIG. 3. The time course of transpiration (A) and photosynthesis (B) from a leaf flap of a banana leaf. The experimental procedure is indicated in C. The light intensity used was 2,000 ft-c and the rate of flow through the chamber was 1 liter min^{-1}.

ture of the air stream was recorded after it had passed through the leaf chamber; C, a 105 minute period of light of a given intensity during which the CO_2 content and dew point temperature of the outflowing air stream was recorded; D, a 30 minute period of darkness during which the CO_2 content and dew point temperature of the outflowing air from the leaf chamber was recorded, and E, finally, another 15 minute dark period during which the incoming air was again sampled.

In most of the experiments the rate of the air flow through the system was maintained at 500 ml/minute, except at the higher light intensities when the flow rate was increased to 1,000 ml/minute to prevent the response from going off scale. A flow rate of 500 ml/minute was calculated to give a wind velocity of 0.02 miles per hour and 1,000 ml/minute a wind velocity of 0.04 miles per hour over the leaf surface.

Experimentation & Results

Initially, the first type of chamber enclosing both the upper and lower surface as well as the two torn edges of the leaf flap was used. Several different light intensities were employed. The results from one such experiment are shown in figure 3. During the first and last 15 minutes the incoming air had a dew point of 56° F, corresponding to a relative humidity

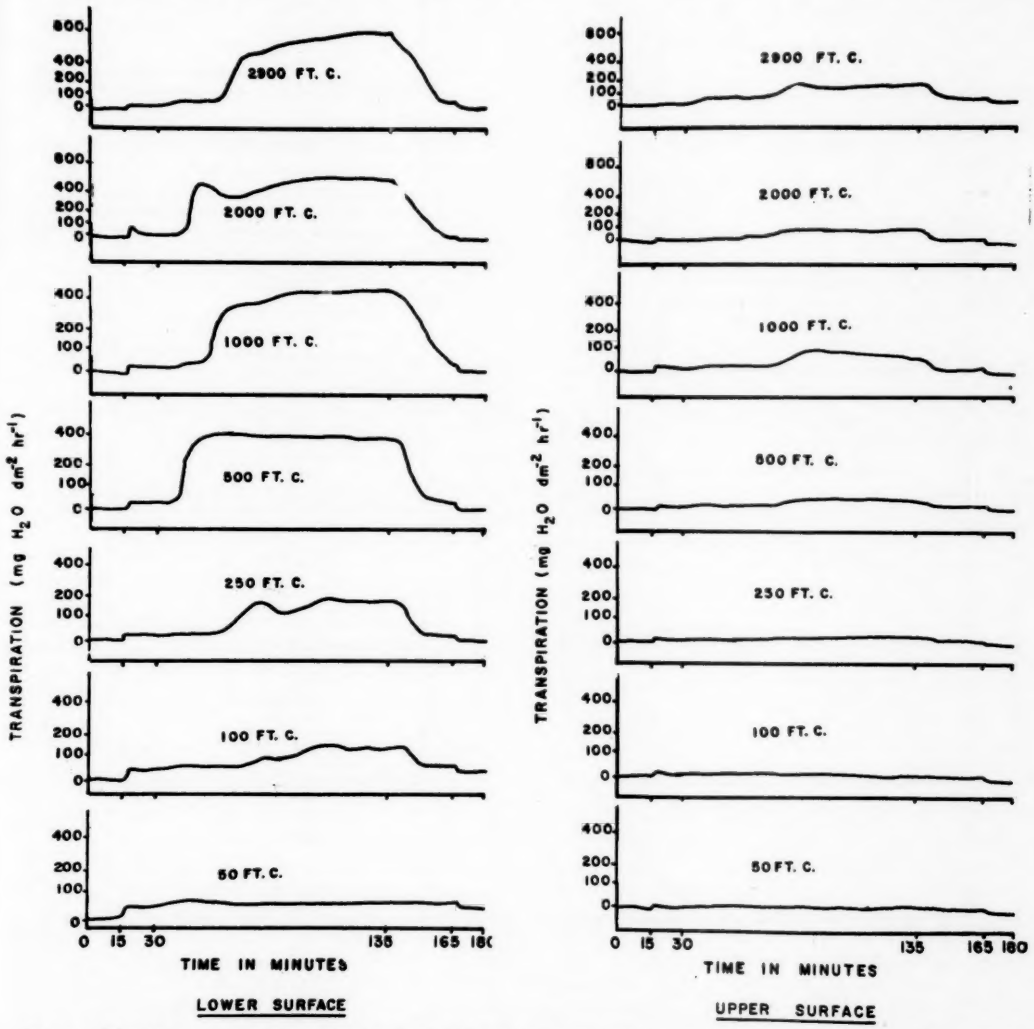

Fig. 4. The time course of transpiration from 100 cm² of upper and lower surface of intact banana leaf exposed to seven different intensities of white fluorescent light. The experimental procedure was as indicated in figure 3 C. The rate of air flow was 1 liter minute^{-1} in the 2,900 and 2,000 ft-c experiments, and 0.5 liter minute^{-1} at the lower light intensities.

of 36 % [curve A (transpiration)]. Starting at time 15, the outflowing air from the leaf chamber (in darkness) was sampled and an apparent transpiration rate of 226 mg of water dm^{-2} $hour^{-1}$ was found. This water loss represents either cuticular transpiration or transpiration from the torn edges of the leaf flap, or both.

Turning on the lights (2,000 ft-c) at time 30 caused a slight increase in transpiration rate, possibly due to an increased leaf temperature. Twenty minutes after turning on the lights there was a sharp rise in the transpiration rate which then leveled off at 943 mg of water dm^{-2} $hour^{-1}$. At time 135 the lights were turned off causing a gradual decline in transpiration rate. At time 165 when the incoming air was again sampled its humidity was the same as during the first 15 minutes of the experiment.

Figure 3 curve B shows the time course of photosynthesis of the same leaf flap during the same experiment. During the first and last 15 minutes of the experiment the incoming air contained 290 ppm CO_2. This was then taken as O on the photosynthesis axis. From time 15 to 30 when the outflowing air from the leaf chamber (in darkness) was sampled, a respira-

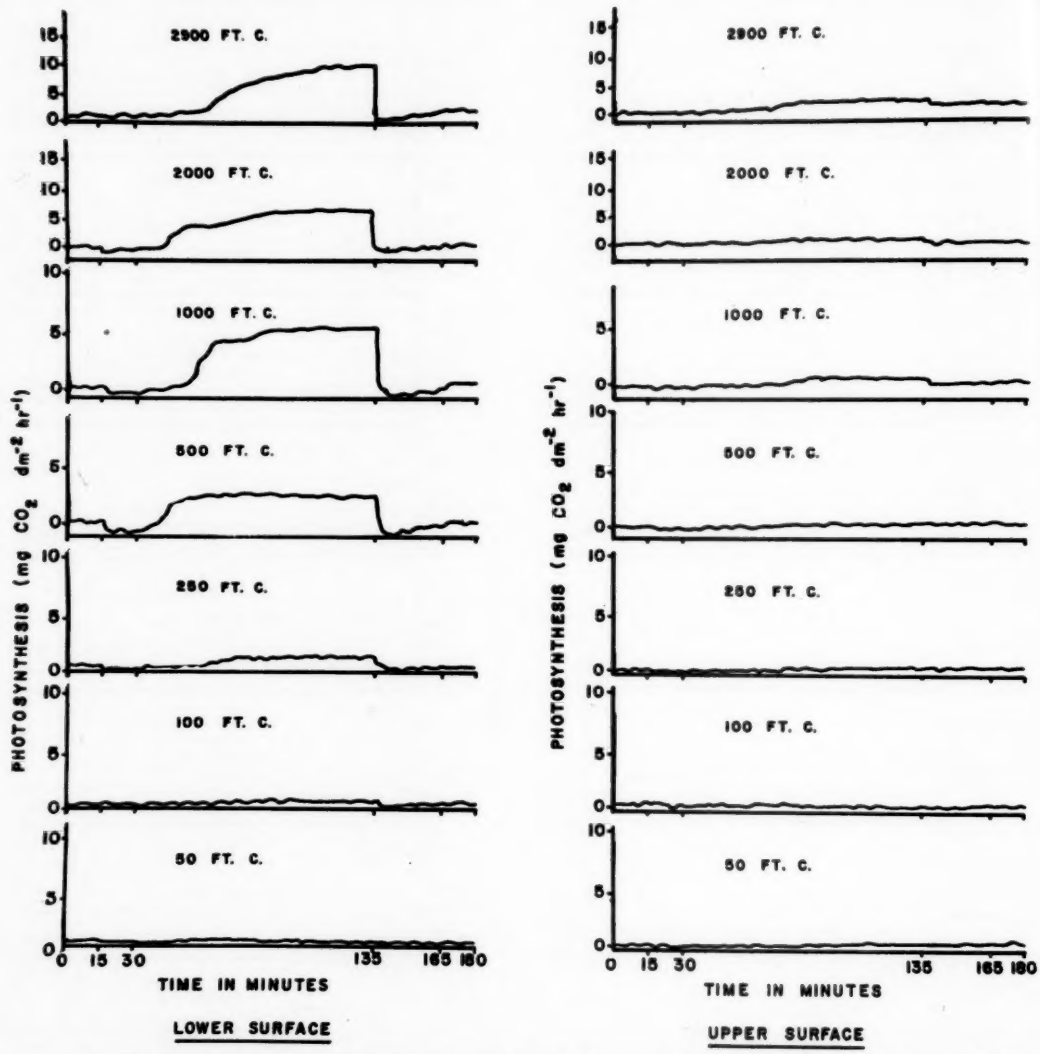

FIG. 5. The time course of photosynthesis from 100 cm^2 of upper and lower surface of intact banana leaf exposed to seven different intensities of white fluorescent light. The experimental procedure was as indicated in figure 3. The rate of air flow was 1 liter $minute^{-1}$ in the 2,900 and 2,000 ft-c experiments, and 0.5 liter $minute^{-1}$ at the lower light intensities.

tory CO_2 release of 1.8 mg of CO_2 dm^{-2} $hour^{-1}$ was found. Turning on the lights at time 30 caused at first only a very slight rise in the curve. After 20 minutes of illumination, the photosynthetic rate rapidly increased and then leveled off at 12.8 mg CO_2 dm^{-2} $hour^{-1}$. The shape of the photosynthesis curve during illumination was similar to the shape of the transpiration curve during this period. At time 135 when the lights were turned off there was an immediate drop to a negative value indicating a respiratory CO_2 release of 3.4 mg dm^{-2} $hour^{-1}$. During the next 30 minutes, while transpiration was gradually decreasing, the curve gradually rose indicating a gradually decreasing respiratory CO_2 release. At time 165 the incoming air was again sampled and found to have the same CO_2 content as during the first 15 minutes of the experiment.

Results using the second type of leaf chamber consisting of two halves, each covering 100 cm^2 of upper or lower leaf surface, respectively, are given in figures 4 and 5. The chamber did not enclose any torn leaf edges. The same standard procedure as shown in figure 3 C was used and the experiment was done at seven different light intensities ranging from 2,900 ft-c to 50 ft-c. All light intensity readings were made on the light incident upon the upper leaf surface with the leaf in its normal horizontal position.

Figure 4 shows the time course transpiration from the upper and lower leaf surfaces at the seven different light intensities. There was a small amount of cuticular transpiration occurring from both surfaces as evidenced by the rise in the curve at time 15 when the air stream was first passed over the leaf in darkness. Likewise, cuticular transpiration was evidenced by a drop in the curve at time 165 when the air stream was switched from the leaf in darkness to incoming air. The average combined cuticular transpiration rates from these separate upper and lower leaf surface chambers amounted to 39 mg of water dm^{-2} $hour^{-1}$ as compared to the 226 mg dm^{-2} $hour^{-1}$ obtained with the first type of leaf chamber which included the two torn edges.

When the lights were turned on at time 30, there was, in those cases where stomatal transpiration occurred, a considerable delay in its onset. When the lights were turned off at time 135, there was a gradual drop in stomatal transpiration. The magnitude of the stomatal transpiration from the lower leaf surface was always much greater than from the corresponding upper surface. Figure 6 shows the maximum rates of stomatal transpiration obtained from the two leaf surfaces at different light intensities.

Figure 5 shows the photosynthesis curves corresponding to the transpiration curves in figure 4. Some respiratory activity is evidenced in some of the curves (lower surface at 2,000, 1,000, 500, & 250 ft-c) by a slight dip at time 15 when the air stream is first passed over the leaf. Following the onset of illumination at time 30, some time elapses before photosynthetic CO_2 fixation is evidenced by a decided rise in the curve. The time lapse before photosynthetic CO_2 uptake becomes evident is, in each case, closely correlated with the time lapse before stomatal transpiration becomes evident (fig 4). During illumination, the shape of the photosynthesis curve is, in each case, similar to the shape of the corresponding transpiration curve in figure 4. The magnitude of apparent photosynthesis from the lower leaf surface was always much greater than from the corresponding upper surface. Figure 6 shows the maximum rates of apparent photosynthesis obtained from the two leaf surfaces at the different light intensities.

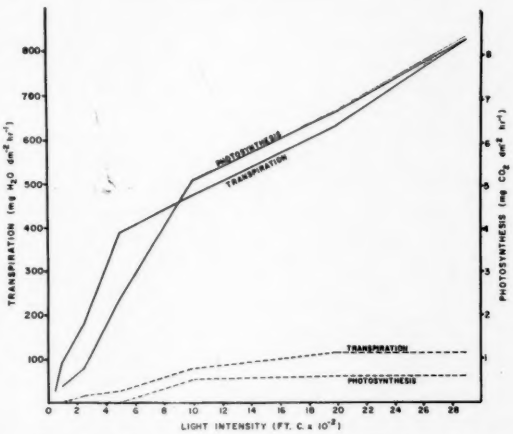

FIG. 6. Maximum rates of photosynthesis and transpiration from the upper surfaces (broken lines) and lower surfaces (solid lines) of banana leaves at different light intensities.

When the lights were turned off at time 135, photosynthesis stopped abruptly giving way to a respiratory CO_2 release which at first was rather pronounced, but which gradually decreased during the time when stomatal transpiration was gradually decreasing (fig 4).

The stomatal frequencies on the two surfaces in question were determined microscopically and found to be 345 stomates cm^{-2} on the upper surface and 1,428 stomates cm^{-2} on the lower surface.

DISCUSSION

By comparing the apparent cuticular transpiration rates obtained in the two types of leaf chambers used, it can be seen that there is considerable water loss through the edges of tears in the leaf blade. Such tears, caused by wind action, are universally present in large numbers on the leaves of field grown banana plants. According to Skutch (21), the internal tissue exposed by such a tear becomes protected against excessive drying by suberization of the tissue along the wound. In the experiments reported here such suberization would scarcely have had time to occur.

A time lapse between the onset of illumination and the beginning of any appreciable photosynthesis or

transpiration is evident in all of the curves presented. This is undoubtedly caused by a delay in stomatal opening. Heath (11) has observed such a delay in the stomatal opening in onion leaves following the onset of illumination. He suggests that this is due to the large central cavity in the onion leaf accumulating large amounts of carbon dioxide during darkness, so that following illumination, it takes some time for the photosynthetic activity of the leaf to reduce the carbon dioxide concentration to a point where stomatal opening becomes possible. Since the banana leaf blade contains some 50 % air space (20), one might propose the same explanation of the time lag, as does Heath concerning the onion leaf. Were this the right explanation, however, the length of the time lag should decrease with increasing light intensity. This does not seem to be the case.

The difference in the magnitude of photosynthesis and transpiration from the two leaf surfaces is undoubtedly a reflection of the stomatal frequencies on the two surfaces. The apparent difference in the light intensity required to cause stomatal opening on the two surfaces is, however, unexplained. This difference becomes even more striking when one considers that all of the light intensity measurements were made on the light incident upon the upper surface. The light actually reaching the lower surface, that is, the sum of the light incident from below and the light transmitted through the leaf from above, was found to be only 7 to 10 % of that incident upon the upper surface.

The shapes of the photosynthesis and transpiration time curves from either surface at any given light intensity are remarkably similar. This would seem to indicate that both of these processes are limited by the stomatal diffusion resistance or the degree of stomatal opening.

Summary

A method of continuously and simultaneously measuring the rates of photosynthesis and transpiration from the upper and lower surfaces of intact portions of banana leaves is described.

It was found that appreciable transpiration occurs from the edges of tears in the leaf blade such as occur almost universally in field grown plants.

The magnitudes of stomatal transpiration and photosynthesis occurring through the upper and lower leaf surfaces were markedly different, as would be expected from the stomatal frequencies on the two surfaces. The light intensities required to initiate stomatal opening on the two surfaces were also different, with the upper surface requiring much higher light intensities than the lower.

Following the onset of illumination, a lag of at least 15 minutes was found to occur before commencement of photosynthesis or transpiration. The length of the lag was not correlated with light intensity. Following the commencement of stomatal transpiration and photosynthesis, the shapes of the curves describing the time courses of the two processes were similar, indicating that both processes were limited by the degree of stomatal opening.

Acknowledgment

I wish to thank Mr. Joseph Baur for his assistance in carrying out most of the experiments here reported.

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REACTION OF GIBBERELLIC ACID & GIBBERELLINS WITH FOLIN-WU PHOSPHOMOLYBDIC ACID REAGENT & ITS USE FOR QUANTITATIVE ASSAY[1, 2]

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Accelerated interest in gibberellic acid and its myriad effects on plant physiological and metabolic responses urge the availability of simple and rapid chemical means of analysis for this versatile plant growth stimulant (14). Analytical methods proposed (1, 2, 7, 9, 15) involve the use of expensive apparatus and, therefore, are not yet applicable to certain routine problems like the stability of gibberellic acid and gibberellin to pH and to autoclaving (8). As an approach to the situation, the reduction of phosphomolybdic acid to molybdenum blue, first noted on paper chromatograms by Simpson (12), has been quantitatively standardized. The method has found practical application in monitoring gibberellin solutions autoclaved in buffers of various pH levels and in correlating the chemical response and biological activity of autoclaved solutions of the chemical (unpublished data, J. H. M. Henderson, & H. D. Graham, Correlation of chemical & biological determinations for gibberellic acid).

MATERIALS & METHODS

REAGENTS: Molybdic acid, 85 % MoO_3—Fisher certified A.C.S. grade.

Sodium tungstate, 10 % sodium tungstate solution (Folin & Wu)—Fisher Scientific Co.

Sodium hydroxide, pellets—certified A.C.S. grade. *O*-phosphoric acid, certified A.C.S. grade—Fisher Scientific Co.

Phosphomolybdic acid reagent—this was prepared as outlined by Folin and Wu (6). Thirty-five grams of molybdic acid and 5 grams of sodium tungstate were added to a 1-liter beaker. Two hundred cubic centimeters of 10 % sodium hydroxide were added and then 200 cc of distilled water. The contents of the flask were boiled vigorously for 40 minutes so as to remove any ammonia present in the molybdic acid. After cooling, the mixture was diluted to 350 cc with distilled water, and 125 cc of concentrated (85 %) phosphoric acid was added. Finally, the mixture was transferred to a 500 cc volumetric flask and made up to volume with distilled water.

Gibberellic acid—Eli Lilly and Co. and Abbott Laboratories.

Gibberellin—Charles Pfizer and Co., Inc.

Gibrel-potassium salt of gibberellic acid—Merck and Co., Inc.

Gibberellin A_1—Abbott Laboratories.

Allogibberic acid—prepared according to the method of Brian et al (4).

Gibberic acid—prepared according to the method of Stodola (13, p 68).

[1] Received November 26, 1960.

[2] A portion of this research was supported by the Brown-Hazen Fund, Research Corp.

TABLE I

INFLUENCE OF VARIABLES ON COLOR DEVELOPMENT IN DETERMINATION OF GIBBERELLIC ACID BY FOLIN-WU PHOSPHOMOLYBDIC ACID REAGENT

VARIABLE	O.D. AT 780 mμ		VALUE OR CONDITION SELECTED FOR REPRODUCIBILITY
Amount of Folin-Wu phosphomolybdic acid reagent added (ml)			
0	0.033		
1	0.197		
5	0.297		
10	0.439		15 ml
12	0.690		
13	0.731		
14	0.733		
15	0.735		
20	0.745		
Temperature of heating (°C)			
40	0.086		
50	0.146		
60	0.201		
70	0.280		100° C
80	0.386		
90	0.523		
100	0.733		
Duration of heating (min)			
0	0.032		
1	0.143		
2	0.276		
5	0.312		
10	0.362		60 min
15	0.426		
20	0.469		
30	0.538		
40	0.611		
60	0.733		
90	0.870		
Color stability (Time lapse, hr)	*Stoppered*	*Unstoppered*	
0	0.731	0.726	
0.5	0.733	0.735	
1.0	0.735	0.731	Measurements
2.0	0.745	0.721	made 10 min after
4.0	0.740	0.721	making up to
8.0	0.723	0.717	volume
12.0	0.721	0.712	
24.0	0.717	0.707	
Nature of diluent			
Distilled H_2O		0.721	
		0.735	Avg.
		0.731	0.729
		0.731	
		0.740	Avg. Distilled
85 % H_3PO_4		0.735	0.735 water

TABLE II

RELATIONSHIP BETWEEN CONCENTRATION OF GIBBERELLIC ACID & GIBBERELLIN & COLOR INTENSITY*

PRODUCT	FINAL CONC.	O.D. IN FOLIN-WU PHOSPHOMOLYBDIC ACID REAGENT AT	O.D. IN FOLIN-WU PHOSPHOMOLYBDIC ACID REAGENT MINUS SODIUM TUNGSTATE AT
Gibberellic acid	(μg/ml)	780 mμ	660 mμ
	0	0.000	0.000
	5	0.105	...
	10	0.174	0.043
	20	0.338	0.095
	30	0.493	0.137
	40	0.665	0.186
	50	0.722	...
	60	...	0.267
	80	...	0.372
Gibberellin	0	0.000	0.000
	10	0.105	0.051
	20	0.240	0.111
	30	0.378	0.169
	40	0.491	0.227
	50	0.658	0.276

* All values corrected for O.D. contributed by reagent blank.

EQUIPMENT: Coleman Universal Spectrophotometer, Model 14. Volumetric pipettes. 25 ml pyrex volumetric flasks. Water bath with stirrers or suitable oil bath. 110° C thermometers.

PROCEDURE: Quantities of gibberellic acid or gibberellin, calculated to give the final concentrations shown in table II, were pipetted into 25 ml volumetric flasks. Fifteen milliliters of the phosphomolybdic acid reagent were added and the contents were thoroughly mixed. The flasks were then placed in a boiling water bath for exactly 1 hour. Zero time was taken as 2 minutes after immersion of the flasks. At the end of the heating period the flasks were rapidly immersed in an ice water bath. After cooling to room temperature, the contents of each flask were made up to volume with distilled water and the intensity of the color developed was measured at 780 mμ with a Coleman Model 14 Spectrophotometer, using distilled water as the blank. A plot of the absorbance (O.D.) as a function of the wave length established the wave length of maximum absorption as indicated in figure 1.

If the sodium tungstate is omitted from the reagent, measurement must be made at 660 mμ. For the assay of unknown samples the procedure is exactly the same as outlined above. The quantity of gibberellic acid or gibberellin present is determined from a standard curve. Any absorbance due to the reagents themselves was accounted for by including, in each determination, duplicate flasks containing all the ingredients except the gibberellic acid or gibberellin. The average optical density of such systems was

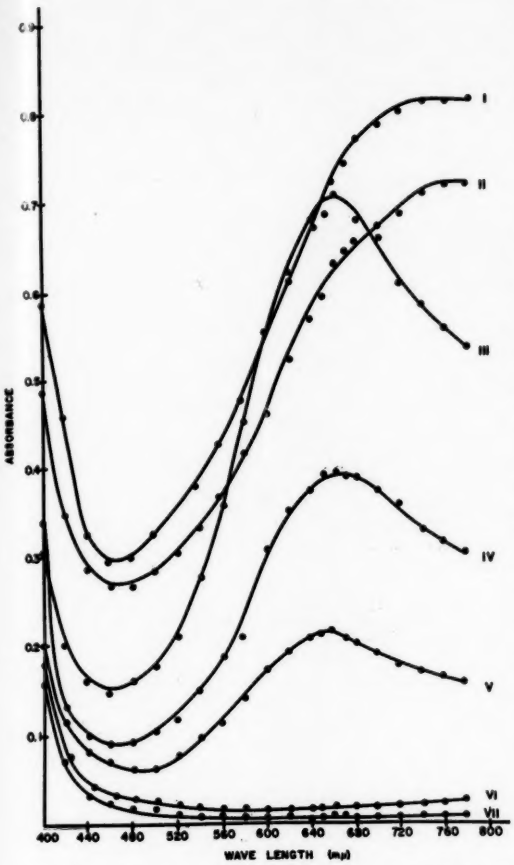

FIG. 1. Absorption spectra of the color produced when gibberellic acid and gibberellin are heated in phosphomolybdic acid of the Folin-Wu phosphomolybdic acid reagent. I = K-gibberellate (80 μg/ml) in Folin-Wu phosphomolybdic acid reagent. II = gibberellin (60 μg/ml) in Folin-Wu phosphomolybdic acid reagent. III = gibberellin (120 μg/ml) in Folin-Wu phosphomolybdic acid reagent minus sodium tungstate. IV = gibberellic acid (80 μg/ml) in Folin-Wu phosphomolybdic acid reagent minus sodium tungstate. V = K—gibberellate (20 μg/ml) in Folin-Wu phosphomolybdic acid reagent minus sodium tungstate. VI = Blank—Folin-Wu phosphomolybdic acid reagent. VII = Blank—Folin-Wu phosphomolybdic acid reagent minus sodium tungstate.

subtracted from all values obtained to give corrected values, as shown for the standard curve data in table II.

RESULTS & DISCUSSION

The Folin-Wu phosphomolybdic acid reagent can be adapted to the quantitative estimation of gibberellic acid, gibberellins, and degradation products of gibberellic acid such as allogibberic acid and gibberic acid. Since potassium gibberellate, a water soluble product, and gibberellic acid dissolved in a small quantity of ethyl alcohol gave positive tests of similar magnitude, it is improbable that the alcohol played any part in the color development.

Data on the influence of several variables on color development are summarized in table I. The final concentration in all cases was 40 μg/ml (total volume 25 ml), and unless otherwise specified, the general procedure was followed. Since gibberellic acid and gibberellin solutions are often made up in buffers, the influence of various salts on color development was investigated. Of the salts tested, the respective maximum final concentrations (M) causing no marked interference with a final gibberellic acid concentration of 40 μg/ml were as follows: sodium chloride, 0.12; boric acid, 0.08; potassium chloride, 0.12; potassium dihydrogen phosphate, 0.08; sodium acetate, 0.05, and potassium acid phthalate, 0.04. At this level of gibberellic acid, a final concentration of 5 μg/ml of indoleacetic acid does not interfere materially. 2,4-D does not respond to the test.

As prepared, the medium is acidic and, under the specified conditions of the test, gibberic acid would be the expected end product. Strong support for this view rests in the specifications for the preparation of allogibberic acid and gibberic acid (13). When the pH of the medium was raised, color development was grossly impaired or completely inhibited. This indicates the necessity of a highly acidic medium, a condition conducive to the production of gibberic acid. The possibility also exists that allogibberic acid could be formed but, under the conditions of the test, this would isomerize to gibberic acid (3, 10, 14). Gibberellin A_1 is probably converted to gibberellin C_1, a ketonic compound (10) which, on a structural basis, should also give the test. Possible participation in the production of the molybdenum blue color by such compounds as epigibberic acid (13) and gibberellenic acid, a precursor of allogibberic acid (13), must also be taken into consideration.

SUMMARY

The proposed method has been found to give reproducible and quantitative results for concentrations of gibberellic acid of 5 to 50 μg/ml and for concentrations of gibberellin of 10 to 50 μg/ml. Within these concentrations, the Lambert-Beer law holds well, especially for gibberellic acid.

Although the method has been applied to only five forms, it is quite likely that any or all of the gibberellin A and C series (10) can be similarly assayed. This conclusion is based on the observation that allogibberic acid and gibberic acid gave positive tests, and on the assumption that the mechanism of the re-

action is the same irrespective of the form of gibberellin being tested.

Since amino acids and sugar will respond to the test, the method is restricted to assays of pure solutions of gibberellic acid and gibberellins. If analysis of mixed systems is contemplated, previous separation and purification of the gibberellic acid or gibberellin must be done and, to assure highly reproducible results, it is advisable to prepare a standard curve for each new batch of reagents.

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THE REDUCTIVE PENTOSE PHOSPHATE CYCLE III.[1] ENZYME ACTIVITIES IN CELL-FREE EXTRACTS OF PHOTOSYNTHETIC ORGANISMS

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The reductive pentose phosphate cycle (6, 29, 8) is currently believed to be the pathway by which CO_2 is converted to carbohydrate in photosynthesis. Evidence for the functional role of this cycle is derived mainly from isotope experiments. Brief exposure of photosynthesizing algae to $C^{14}O_2$ produces radioactive 3-phosphoglycerate (3-PGA)[3], labelled mainly in the carboxyl group (11). The cyclic nature of the photosynthetic carbon path is indicated by the subsequent appearance of isotopes in the α and β positions of 3-PGA, as well as in phosphate esters of ribulose and sedoheptulose (28). Kinetic studies by Calvin and Massini (14) and Bassham et al (7) on the fluctuation of pool sizes, revealed that transition from light to dark resulted in an increased concentration of 3-PGA accompanied by a decreased concentration of sugar diphosphate. Studies (43) carried out with algae subjected to changing partial pressures of CO_2 demonstrated a fall in 3-PGA and a rise in ribulose diphosphate (RDP) concentration when the CO_2 concentration was changed from 1 % to 0.003 %.

Enzymes of the pentose phosphate cycle are ubiquitously distributed (2, 4, 5, 17, 18, 20, 21, 22, 23, 24, 28, 29, 30, 36, 37, 38, 41). However, no single photosynthetic organism has been shown to possess all of the requisite enzymes and in amounts sufficiently high to support photosynthesis by this pathway. In this report, the quantitative aspects of all the pentose phosphate cycle enzymes in extracts of several photosynthetic organisms were investigated. We compared the rate of photosynthetic CO_2 fixation of the intact organism with the enzyme capacities (activities at saturating substrate concentration) in an extract of the same organism. Our results indicate that several enzymes of the pentose phosphate cycle appear to be insufficiently active to satisfy the rate of CO_2 fixation in the intact organism.

MATERIALS & METHODS

CULTURE OF ALGAE: A, Cultures of the thermophilic *Chlorella pyrenoidosa* Chick. strain 7-11-05 (33) were obtained from Dr. Dean Burk. The cells were grown in 500 ml gas wash bottles. Air containing 5 % CO_2 was bubbled through the bottles while they were gently shaken to keep the algae suspended. Illumination of 1,000 to 2,000 ft-c was supplied by a 150 w incandescent bulb placed about six inches from the culture bottles. At this site the temperature was maintained at 38° C. The medium was that recommended by Burk (personal communication). A liter of medium contained: $MgSO_4 \cdot 7H_2O$, 5.0 g; KH_2PO_4, 2.5 g; NaCl, 2.0 g; urea, 0.4 g; sequestrene-Na · Fe, 4.0 mg; $CaCl_2$, 22.0 mg; $CuSO_4 \cdot 5H_2O$, 0.158 mg; H_3BO_3, 5.7 mg; $MnCl_2 \cdot 4H_2O$, 3.6 mg; $ZnSO_4$, 0.44 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.035 mg; $NaVO_3$, 1.92 mg; the medium was adjusted to pH 5.7 with NaOH. The cells were harvested after growth overnight, washed at 3,000 × *g* at 20° C with 0.05 M tris buffer, pH 7.4. A pure culture of the same strain was also obtained from Dr. C. Sorokin and grown as described (34).

B, A slant of a low temperature *Chlorella pyrenoidosa* was kindly supplied by Dr. M. Gibbs. The cells were grown in 1 liter Erlenmeyer flasks in the medium of Norris et al (25), except that 10 mg NaCl per liter were added, and 5 mg $FeSO_4$ and 2 mg tartaric acid per liter were substituted for the suggested Fe-EDTA additions. The container was stoppered with a cotton plug through which a bubbling tube was inserted. Air containing 5 % CO_2 was bubbled through the medium under sterile conditions. The temperature was maintained at 23° and illumination at 1,000 ft-c. The cells were washed as described above.

C, A culture of *Euglena gracilis* Klebs., strain Z, was obtained from Dr. S. Hutner. The organisms were grown under sterile conditions in the medium of Cramer and Myers (16) at 24° C, and illumination was supplied by fluorescent lamps at an intensity of 400 ft-c. Cells were harvested after 6 days and washed at 1,000 × *g* at 2° C with 0.1 M tris, pH 7.4, containing 0.12 % EDTA.

[1] Received December 5, 1960.

[2] Predoctorate Fellow, National Cancer Institute, National Institutes of Health, Bethesda, Md. Present address: National Institutes of Health, Bethesda, Md. The experimental work presented in this paper was taken in part from a doctoral dissertation submitted to the faculty of New York University.

[3] Abbreviations: DPN = diphosphopyridine nucleotide. EDTA = ethylenediamine tetraacetate. FDP = fructose-1, 6-diphosphate. G-3-p = glyceraldehyde-3-phosphate. GSH = glutathione. 3-PGA = 3-phosphoglycerate. Pi = inorganic phosphate. R-5-p = ribose-5-phosphate. RDP = ribulose 1,5-diphosphate. SDP = sedoheptulose-1, 7-diphosphate. TPN = triphosphopyridine nucleotide. tris = tris (hydroxymethyl) aminomethane. Xu-5-p = xylulose-5-phosphate.

PREPARATION OF CELL-FREE EXTRACTS: A homogenate from fresh spinach leaves was prepared by the method of Arnon (3).

Extracts of Euglena and Chlorella were prepared in a Nossal shaker (26). Seven milliliters of 0.3 mm diameter glass beads (Minnesota Mining & Mfg. Co.) and the cell suspension in a volume of 10 ml were dispensed into the Nossal cup. The cup was evacuated (27) and shaken at 2° C for 10-second intervals, separated by 1 minute periods of cooling in an ice bath. Three shaking periods were sufficient to effect complete breakage of Euglena as indicated by microscopic observation. Four 10-second periods of shaking were used for Chlorella. After treatment in the Nossal shaker, the preparations were centrifuged for 10 minutes at 1,000 × g to free the suspensions of glass beads and cell debris.

REAGENTS: $Na_2C^{14}O_3$ solution was obtained from Tracerlab, Inc. Sequestrene–Na · Fe was obtained from the Alrose Chemical Co., Cranston, R.I. All other reagents and auxiliary enzymes were obtained as described previously (29, 30, 44, 15). Before use all barium salts were dissolved and passed through a Dowex-50 Na^+ column to remove the barium.

ASSAY OF ENZYMES, CO_2 FIXATION, & DETERMINATION OF CHLOROPHYLL: RDP carboxylase was determined either spectrophotometrically (29) or by a $C^{14}O_2$ fixation assay similar to that of Jakoby et al (24). In a volume of 0.5 ml, the following were added: tris buffer, pH 7.8, 20 μmoles; GSH, 2 μmoles; bovine serum albumin, 0.4 mg; $MgCl_2$, 4 μmoles; $KHC^{14}O_3$, 30 μmoles; RDP, 0.40 μmoles, and the extract to be assayed. After a suitable incubation period, 0.5 ml of 10 % trichloroacetic acid was added and an aliquot of the centrifuged supernatant solution was plated and counted.

Glyceraldehyde–3–P dehydrogenase and 3–phosphoglycerate kinase were assayed in the back direction (44). Transaldolase (15), xylulose–5–P epimerase and ribose–5–P isomerase (36), phosphoribulokinase (29), aldolase (44), glyceraldehyde–3–P isomerase (10), fructose diphosphatase, and sedoheptulose diphosphatase (30) were measured as described in the references. Transketolase was determined as previously (15) except that 10 μg of crystalline glyceraldehyde–3–P isomerase and α–glycerophosphate dehydrogenase were substituted for the 52 to 72 % ammonium sulfate fraction of rabbit muscle. All enzyme assays were carried out in either tris or glycylglycine buffer between pH 7.4 and 7.8.

Chlorophyll of spinach leaves and of Chlorella was determined by the method of Warburg (cf 39). Chlorophyll of Euglena and of spinach homogenate were determined by the method of Arnon (1). Rates of photosynthesis of intact cells were determined by fixation of radioactive bicarbonate. The experiments were carried out in a modified Warburg apparatus in which illumination was provided by a bank of fluorescent lamps mounted under the glass bottom of the tank providing 400 ft-c to the reaction vessels. This was increased to 1,000 to 2,000 ft-c by overhead illumination from 500 w incandescent bulbs. A cooling unit and heating bar provided a wide range of temperature control. The vessels were shaken in an air atmosphere.

RESULTS

CO_2 FIXATION OF INTACT ORGANISMS: Experiments were carried out with intact spinach leaf squares to ascertain the rate of CO_2 fixation obtainable in this tissue. Washed spinach leaves were destemmed and the midribs removed. The remainder of the leaf was cut into 1 cm squares. Three such squares were incubated in a 3 ml volume with NaCl (0.035 M), tris buffer (0.013 M, pH 7.4) and radioactive bicarbonate. Bicarbonate saturation was reached at 0.03 M. Experiments were carried out for 15 minutes at 20°. After terminating the experiment with 5 % trichloroacetic acid, the leaf squares and suspending medium were washed into a Potter-Elvejhem type homogenizer and ground to a uniform suspension. An aliquot of the suspension was transferred to a planchet, dried, and counted. With 30 μmoles bicarbonate per ml (about 10,000 counts/min per μmole) CO_2 fixation was 32 μmoles/mg chlorophyll/hour.

Washed Euglena were incubated with tris buffer (0.04 M, pH 7.4) and radioactive bicarbonate for the measurement of CO_2 fixation. The activity was relatively constant throughout a concentration range of bicarbonate of 1×10^{-3} M to 1.7×10^{-2} M. At higher concentrations, the rate of CO_2 fixation increased. The plateau region at the lower bicarbonate concentrations suggests that Euglena may have a concentrating mechanism for bicarbonate which is saturated at these levels. At a bicarbonate concentration of 1×10^{-2} M and illumination of 1,000 ft-c, the rate of CO_2 fixation at 20° was 100 μmoles/mg chlorophyll/hour. Under these conditions, the CO_2 fixation activity was light dependent, proportional to time and to the amount of Euglena added, at least up to a concentration of 0.3 mg chlorophyll/ml.

Washed cells of the thermophilic Chlorella, 7–11–05 (33) were incubated with radioactive $NaHCO_3$ at pH 7.4, tris 0.04 M. A light-dependent CO_2 fixation proceeding at a constant rate for at least one hour and proportional to cell concentration at low chlorophyll concentrations could be demonstrated. The carboxylation activity was saturated with bicarbonate at a concentration of 2×10^{-2} M. Rates of photosynthesis measured under these conditions ranged from 200 to 400 μmoles CO_2/mg chlorophyll/hour at 38° C. These rates, as determined by $C^{14}O_2$ fixation were found to be in satisfactory agreement with measurement of net CO_2 uptake.

The normal low temperature strain of *Chlorella pyrenoidosa* exhibited properties of CO_2 fixation essentially similar to that of the thermophilic organisms. At a bicarbonate concentration of 1×10^{-2} M and illumination of 1,000 ft-c the rate of CO_2 fixation was 150 μmoles/mg chlorophyll/hour at 23° C.

TABLE I

PENTOSE PHOSPHATE CYCLE ENZYME ACTIVITIES IN EXTRACTS OF PHOTOSYNTHETIC ORGANISMS

ENZYME	ACTIVITY (μmoles/mg CHLOROPHYLL/hr)*			
	SPINACH HOMOGENATE	EUGLENA	CHLORELLA (HIGH TEMP.)	CHLORELLA (LOW TEMP.)
3-PGA kinase	4,500	5,875	12,590	2,130
G-3-P isomerase	1,060	2,540	4,913	3,700
G-3-P dehydrogenase (DPN)	390	334	406	260
G-3-P dehydrogenase (TPN)	180	280	368	170
Aldolase	300	68	312	99
Phosphoribulokinase	600	107	1,133	227
Transketolase	300	98	214	73
R-5-P isomerase	508	169	664	196
Xu-5-P epimerase	1,630	360	1,584	22
RDP carboxylase	150	45	57	21
FDPase	39	13	21	22
SDPase	8	1.5	4	30
Transaldolase	6	4.5	1.2	1
CO_2 fixation in intact cells	32	100	259	150
Temp. ° C	20°	20°	38°	23°

*Calculated for temperature indicated in bottom line.

Fixation of CO_2 in the dark was well below 10 % of that in the light in spinach leaves, Euglena, and Chlorella.

ENZYME ACTIVITIES IN CELL-FREE EXTRACTS: In table I are listed representative activities of the pentose phosphate cycle enzymes in cell-free extracts of the four photosynthetic organisms examined. The enzyme capacities are expressed as μmoles of substrate converted to product in 1 hour by the amount of extract derived from organisms containing 1 mg chlorophyll. For comparative purposes all enzyme rates were corrected to the indicated temperature assuming a temperature coefficient of 2. It should be noted that in all cases the activities of RDP carboxylase, FDPase, SDPase, and transaldolase are lower than required to support the rate of CO_2 fixation of the whole cells. In spinach, only transaldolase and SDPase appear low.

Although some variations have been observed in the enzyme activities in extracts, e.g. from different batches of spinach, the recorded values are quite representative. For example, in about 12 assays of RDP carboxylase in various spinach samples variations of no more than a factor of 2 were encountered. Somewhat larger variations were noted in transaldolase activity which was too low for very accurate assays. Recovery experiments, e.g. with known transaldolase added to the crude extract, did not indicate the presence of an inhibitor.

It was conceivable that the method of preparing the extract resulted in incomplete release or destruction of enzyme activity. To explore this possibility a kinetic study of enzyme release was carried out (fig 1). A Chlorella suspension was assayed for two pentose phosphate cycle enzymes after successive disruption periods in the Nossal shaker. Three 10-second shaking periods released the maximum amount of RDP carboxylase and aldolase, while additional shaking periods neither released more enzyme activity nor resulted in the destruction of activity. Further-

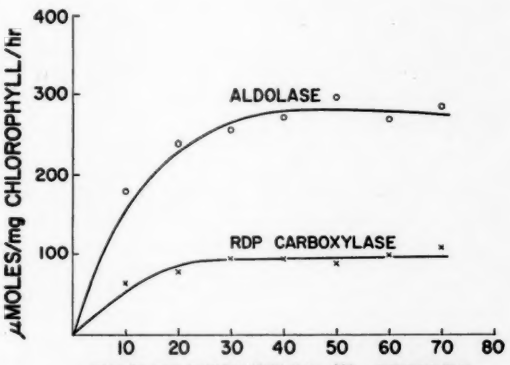

FIG. 1. Time course of enzyme release from chlorella 7-11-05 by disruption in a Nossal shaker. A suspension of Chlorella 7-11-05 was dispensed into a Nossal cup and subjected to 10 second periods of shaking. An aliquot was removed before shaking was begun and after each shaking interval. The samples were assayed for aldolase and RDP-carboxylase ($C^{14}O_2$ fixation assay). The assay temperatures were 26° C for aldolase and 38° C for RDP-carboxylase.

more, a small residue obtained after centrifugation of the disrupted Chlorella did not fix CO_2 nor did it stimulate the CO_2 fixing activity residing in the soluble fraction. Studies of the stability of the enzyme in the extract also failed to reveal any striking inactivation processes.

A closer examination was made of the RDP-carboxylase activity of Chlorella in order to determine whether or not the addition of certain cofactors might result in a marked stimulation of its activity. The addition of the growth medium, or of cobalt, nickel, and manganese, which can substitute for magnesium in spinach RDP-carboxylase (42), were ineffective in Chlorella extract. The enzyme did not exhibit any unusual temperature properties since it had a typical Q_{10} of 2.

A study of the effect of bicarbonate concentration on the RDP-carboxylase activity of Chlorella extract revealed that the activity was being measured at a bicarbonate concentration which resulted in maximal activity. Note that the curve was essentially the same as that obtained in a bicarbonate concentration study for photosynthetic CO_2 fixation of the intact organisms.

The possibility of a transcarboxylase activity of RDP-carboxylase (29) was tested by addition of various potential CO_2 donors. None of a number of intermediates of the urea cycle (ornithine, citrulline, arginine) or Krebs cycle (oxaloacetate, succinate, malate, citrate, isocitrate, glutamate) stimulated CO_2 fixation by the extract in the presence of RDP. None of the factors tested appeared to catalyze appreciable CO_2 fixation in the absence of RDP. ATP, which has previously been observed to inhibit purified spinach RDP-carboxylase (unpublished observations in this laboratory), resulted in a 35 % inhibition of the activity in the crude Chlorella extract.

A survey test of some vitamins, concentrated cofactors from liver or yeast and crude boiled juice of Chlorella revealed no stimulation of the carboxylation of RDP. In all cases, the omission of RDP resulted in no CO_2 fixation.

Discussion

Our studies show that, as with glycolysis (44) and the urea cycle (13), assays for the pentose phosphate cycle enzymes may be applied to crude cell extracts. In analyses of glycolysis, the urea cycle, and pyrimidine biosynthesis, the least active enzyme had a capacity several-fold higher than had the overall process (13, 44–46). On the basis of the observation that cell-free extracts of *Anacystis nidulans* show low aldolase activity, Richter (31) has concluded that the pentose phosphate cycle is not the pathway for hexose synthesis by this organism. Similar observations have been made by Szymona and Doudoroff (35) in a study of the metabolism of *Rhodopseudomonas spheroides*. In the present study of the reductive pentose phosphate cycle, the activities of several enzymes (RDP–carboxylase, fructose diphosphatase, sedoheptulose diphosphatase, transaldolase) are not high enough to satisfy the rate of overall CO_2 fixation observed in intact cells. It should be emphasized that the conditions employed in this work of measuring CO_2 fixation in intact cells in tris buffer are rather unphysiological, particularly in the case of spinach leaves. The conditions were chosen only to allow for a direct comparison with the enzyme assays. The relatively low values of photosynthesis obtained with intact spinach leaves represents therefore, a minimum value. Any increase in the rate of CO_2 fixation under more physiological conditions would make the discrepancy with the enzyme assays even greater.

Of the four enzymes that were found particularly low, only transaldolase was measured in the direction opposite to that believed to be operative in photosynthesis. This choice of assay procedure was determined only by the limited availability of erythrose–4–P. In view of the particularly low values for transaldolase observed in extracts of photosynthesizing cells, a few measurements in Chlorella extracts were carried out in the other direction, measuring the formation of glyceraldehyde–3–P from fructose–6–P and erythrose–4–P. Although the reaction proceeded faster in this direction (3–6 μmole/mg chlorophyll/hr) it still represented only a small fraction of the overall CO_2 fixation rate. To eliminate the possibility that an inhibitor in the extract interfered with the assay of these enzymes of low activity, known amounts of purified enzyme solutions were added to the crude extract and reassayed. No evidence for an inhibition was obtained since the recovery of activities was satisfactory.

It is obvious that this type of data, obtained after disruption of the cell structure, is insufficient to eliminate the reductive pentose phosphate cycle as a major CO_2 fixation pathway in photosynthesizing cells. They do, however, suggest that a search should be made for alternative enzymes catalyzing individual steps of the cycle, particularly where apparent enzyme deficiencies exist. For example, a phosphate transfer reaction might substitute for the hydrolytic cleavage of fructose diphosphate to fructose–6–P. Attempts to demonstrate such a reaction with various P–acceptors, such as ribulose–5–P, have been made in our laboratory during the past years without success. For the utilization of erythrose–4–P in the cycle, the transaldolase reaction could be replaced by aldolase followed by a sedoheptulose diphosphatase (32, 9). Since the latter activity is also low in extracts of photosynthesizing cells, phosphate transfer to ribulose–5–P from sedoheptulose diphosphate was explored, again with no success.

An even more difficult problem is the low activity of RDP–carboxylase. It has been pointed out previously (29) that its low affinity for $KHCO_3$ calls for a large excess of this enzyme in photosynthesizing cells. The low RDP-carboxylase content reported in this study in comparison to the overall CO_2 fixation rate further accentuates the discrepancy between enzyme activity and CO_2 fixation rate of intact cells.

In view of this low affinity of carboxylase for CO_2 several investigators have considered the possibility that an activated form of CO_2 is involved in photosynthesis. Since crude extracts were shown to require the same high concentration of bicarbonate for maximal CO_2 fixation into RDP as the purified enzyme (29) we compared the effect of bicarbonate concentration of CO_2 fixation in intact cells and crude extracts (table II). In spinach and Chlorella, the bicarbonate concentration required for saturation in the extract coincides with that in the whole cells. The lower bicarbonate concentration required for saturation of CO_2 fixation in intact Euglena (compared to Euglena extract) may be due to a CO_2 concentrating system. Except for Euglena, therefore, the data speak against an activated form of CO_2 participating in photosynthesis.

Although attempts have been made and described above to rule out enzyme inactivation during the process of mechanical shaking, an alternative procedure of cell disruption, namely sonic disintegration, was employed in some experiments. Breakage of Chlorella was complete after 20 minutes of sonic vibration. Low speed centrifugation of the suspension resulted in almost no residue and was therefore omitted. Such crude suspensions must therefore have included broken cell wall material and any enzymes associated with it. Nevertheless, enzyme assays of RDP-carboxylase, fructose diphosphatase, glyceraldehyde-3-P dehydrogenase, and transaldolase gave values similar to those found in extracts obtained in the Nossal shaker.

The possibility that the pentose phosphate cycle enzymes within the intact cell operate in an organized way, resulting in an increased activity, is not one that is easily tested with the techniques currently available. The measurement of the enzymes in the profiles reported here were carried out at optimal substrate concentrations. Since it is unlikely that such conditions are prevalent in the intact cell, the discrepancy discussed above may be even greater than is immediately apparent from the data.

Numerous other CO_2 fixation pathways have been proposed in the past, particularly some channeling into amino acids (19, 40). However, none of these pathways has been formulated to include a cyclic regeneration of a CO_2 acceptor, a feature which is present in the reductive pentose phosphate cycle. A scheme lacking in this essential point fails to account for the process of photosynthesis.

TABLE II

COMPARISON OF SATURATING CO_2 CONCENTRATIONS IN DIFFERENT TISSUES & EXTRACTS

SYSTEM EXAMINED	SATURATING BICARBONATE CONC (M)
Spinach leaves	3×10^{-2}
Spinach homogenate	3×10^{-2}
Euglena	1×10^{-3}
Euglena extract	3×10^{-2}
Chlorella 7–11–05	2×10^{-2}
Chlorella extract	2×10^{-2}

SUMMARY

Profiles of the enzymes of the pentose phosphate cycle were determined in cell-free extracts of spinach, Chlorella and Euglena. A comparison of these activities with the rate of photosynthetic CO_2 fixation of intact cells revealed marked discrepancies. Certain enzymes of the pentose phosphate cycle appeared to be present in insufficient amounts to support the rate of photosynthesis of the intact cells. Evidence was presented suggesting that the enzymes were completely extracted and not inactivated in the process of preparing cell-free extracts. The significance of these findings is discussed.

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CHLOROSIS IN HIGHER PLANTS AS RELATED TO ORGANIC ACID CONTENT [1, 2]

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In recent years comparative studies have been made between chlorotic plants and healthy plants to determine if any consistent distortion of the normal metabolic pattern occurs irrespective of the cause of chlorosis. Experiments by Iljin (6) indicated a greater amino acid and organic acid content in chlorotic leaves from grape plants grown on calcareous soil than in normal green leaves. DeKock et al (4) also found that significantly larger amounts of free amino acids and organic acids were associated with tissue in which chlorosis was present. Rhodes et al (11) observed that aspartic and glutamic acids which are formed directly from organic acids were present in high concentrations in lime-induced chlorotic bean leaves.

From earlier studies we may assume that the disorders in metabolism associated with chlorosis in higher plants, such as blocks in the pathways for synthesizing amino acids and proteins from organic acids, or abnormal operations of the TCA cycle are either causative factors or the result of chlorosis. In this study, in order to gain more evidence for the mechanisms by which chlorosis is induced, comparative studies on the organic acid fractions between chlorotic and healthy leaves were conducted. Chlorosis was induced in the experimental plants by high phosphorous, manganese, bicarbonate, or iron deficiency.

MATERIALS & METHODS

EXPERIMENTAL PLANTS. *Glycine max* (L.) Merr. variety PI–54619–5–1 (PI) soybean was selected as an experimental plant because of its sensitivity to iron chlorosis by mineral deficiencies or excesses. Hawkeye (HA) soybean was also used in one experiment to compare with the less resistant PI variety.

The soybeans were germinated on cheesecloth in half strength Hoagland's nutrient solution (5), and then transferred to containers having the same nutrient composition. In one experiment the plants were transferred to pots containing pearlite (expanded pumice) and irrigated daily with nutrient solution. When seedlings were about ten days old, they were transplanted to aerated nutrient solutions in growth chambers where light intensity, day length, and temperature were controlled. Plants growing in perlite were not transplanted but irrigated daily with the treatment nutrient solution. Leaves from the plants were collected between 2 and 3 PM on the day of harvest to reduce environmental influences of organic acid content.

CULTURE METHODS. *Iron deficiency induced chlorosis*: The nutrient solution consisted of the following concentration of minerals: (meq/l) Ca 2, Mg 1, K 2.2, NH_4 0.5, NO_3 5.2, SO_4 0.5; (ppm) P 6.0, Mn 0.08, B 0.04, Zn 0.03, Cu 0.01, Mo 0.01, and Cl 5.0. Iron was added to the control plants at the rate of 7.5 ppm and omitted from the iron deficiency treatments; the solution was maintained at pH 5.5 by additions of NaOH or HCl when required. PI soybeans were grown in pearlite (expanded pumice) in liter plastic containers and irrigated daily with nutrient solution as indicated above. Half of the plants received complete solution and half received solutions deficient in iron. Plants were harvested after 6 weeks when chlorosis first became evident. Apparently pearlite contained iron contaminations which delayed the onset of symptoms. Organic acid determinations from this experiment are denoted in table I as sample A.

TABLE I

ORGANIC ACIDS IN LEAVES FROM NORMAL & IRON-DEFICIENT CHLOROTIC PLANTS

SAMPLE TREATMENT ACID fr wt BASIS (meq/g OF LEAF)	A*		B**	
	NORMAL	CHLOROTIC	NORMAL	CHLOROTIC
Fumaric acid	8.8	4.9	3.3	2.4
Succinic	4.9	2.3	19.6***	1.0
Malonic	36.3	16.6		5.6
Malic	29.3	17.4	7.4	6.6
Citric	37.5	43.8	24.2	30.6
Total	116.8	85.0	54.5	46.2
ppm Iron in leaves	54	40	62	44

* Plants were grown in pearlite and irrigated daily with nutrient solution containing 7.5 ppm Fe for normal plants and no added Fe for chlorotic plants.

** Plants were grown in nutrient solutions containing 7.5 ppm Fe for normal plants and no added Fe for chlorotic plants.

*** Combined concentrations of succinic and malonic acids.

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TABLE II

ORGANIC ACIDS IN LEAVES FROM NORMAL & BICARBONATE CHLOROTIC PLANTS

SAMPLE TREATMENT ACID (meq/g OF LEAF) fr wt BASIS	PI A*		PI B*		HAWKEYE C*	
	NORMAL	CHLOROTIC	NORMAL	CHLOROTIC	NORMAL	CHLOROTIC
Fumaric	0.3	0.5	1.6	0.3	1.4	6.8
Succinic	0.3	0.5	0.7	0.3	14.6**	3.2
Malonic	0.5	10.8	8.2	28.0		23.8
Malic	1.2	1.6	21.0	17.7	0.5	23.8
Citric	20.5	26.3	15.2	22.3	2.1	50.2
Total	22.8	39.7	46.7	68.6	18.6	107.8
ppm Iron in leaves	32	16	41	15	160	18

* Plants were grown in three separate experiments in solutions containing 10 meq/l $NaHCO_3$ (chlorotic treatment) and 10 meq/l NaCl (normal treatment); pH of the solution was maintained at 7.8. PI soybean was the experimental plant in samples A and B; HA soybean was the experimental plant for sample C.

** Combined concentrations of succinic and malonic acids.

Plants were also grown in nutrient solution but received the same nutrient solution treatment as sample A. Chlorosis occurred after 3 weeks, at which time the plants were harvested. Experiments from this treatment are denoted as sample B in table I.

Bicarbonate-induced chlorosis: The nutrient solution consisted of the same concentration of minerals as listed for the iron deficiency experiment with the exception of Fe. Iron was added at the rate of 3.5 ppm. For bicarbonate treatments 10 meq/l of $NaHCO_3$ were added and controls contained 10 meq/l of NaCl. In the bicarbonate treatment pH 7.8 was maintained by aerating with 1 % CO_2 in air; controls were maintained at pH 7.8 by daily additions of NaOH. PI soybeans were grown in nutrient solution with bicarbonate treatments as listed above. The organic acid analyses of the leaves of the PI soybeans subjected to the bicarbonate treatment for 10 and 14 days in two separate experiments are found in table II, as samples A and B. Hawkeye soybeans, a more chlorosis resistant soybean than PI, were subjected to the same treatment conditions for 11 days. These results are found under column C in table II.

High phosphorous induced chlorosis: The nutrient solution consisted of the same concentration of minerals as listed for iron deficiency experiments except for Fe and P concentrations. High phosphorous treatments received 10 ppm P and 3.5 ppm Fe; controls contained 0.05 ppm P and 3.5 ppm Fe. The pH of the nutrient solution was maintained at 5.5. Chlorosis was induced in PI soybeans by the high phosphorous treatment. Plants were harvested 20 days after treatment initiation when chlorotic symptoms first became evident. Organic acid analyses are found in table III.

High manganese induced chlorosis: The nutrient solution consisted of the following nutrients: (meq/l) Ca 10, Mg 4, K 5, NO_3 10, SO_4 4; (ppm) P 16, B 0.25, Zn 0.01, Cu 0.01, Mo 0.015, Fe 3.5 and Cl 5. High Mn treatments contained 10 ppm and the controls received 0.25 ppm Mn; pH of the solution culture was maintained at 5.5. Leaves from PI soybeans were harvested 8 and 12 days after treatment initiation in two separate experiments. Organic analyses of these experiments are reported in table IV, under samples A (8 day) and B (12 day).

TABLE III

ORGANIC ACIDS IN LEAVES FROM NORMAL & HIGH PHOSPHORUS CHLOROTIC PLANTS

SAMPLE TREATMENT ACID (meq/g OF LEAF) fr wt BASIS	A*	
	NORMAL	CHLOROTIC
Fumaric		3.5
Succinic	1.0	2.4
Malonic	8.1**	6.2
Malic	3.7	5.2
Citric	17.7	28.4
Total	30.5	45.7
ppm Iron in leaves	100	32

* Chlorosis was induced by growth in nutrient solution containing 10 ppm P at pH 5.5. Normal plants were grown in solutions containing 0.05 ppm P in the growing medium. Nutrient solutions were changed every 5 days.

** Combined concentrations of succinic and malonic acids.

PROCEDURE

The general procedures followed for organic acid determination were similar to those of DeKock and Morrison (4) with certain modifications where necessary.

Twenty-five grams of PI soybean leaf tissue were macerated for 3 minutes in an Omni mixer in an ice bath with 100 ml of 70 % acetone and allowed to stand overnight. After filtration the residue was extracted with hot 0.01 N HCl, filtered, and combined with the first extractions after removal of the acetone by distillation. The pH was then adjusted to 7.0 and the volume of extract reduced to about 150 ml in vacuo at 40° C.

TABLE IV

ORGANIC ACIDS IN LEAVES FROM NORMAL & HIGH MANGANESE CHLOROTIC PLANTS

SAMPLE TREATMENT	A*		B*	
ACID (meq/g OF LEAF fr wt BASIS	NORMAL	CHLOROTIC	NORMAL	CHLOROTIC
Fumaric	9.2	7.4	7.4	6.4
Succinic	32.6**	14.3**	1.0	3.9
Malonic			24.9	12.1
Malic	19.5	15.8	14.0	21.7
Citric	36.2	45.6	21.6	33.6
Total	97.5	83.1	68.9	77.7

* PI soybeans were grown in two separate experiments in nutrient solution containing 10 ppm Mn (chlorotic treatment) and 0.25 ppm Mn (normal treatment) at a constant level of 3.5 ppm Fe.

** Combined concentrations of succinic and malonic acids.

The extract was passed through a permutit Q column (28 cm × 1.5 cm) in which the amino acids were absorbed. The effluent from the permutit Q column was run through an Amberlite IR4B column, which absorbed the organic acids. The effluent was discarded. The organic acids were eluted from the Amberlite column with 200 ml of a N-NH_3 solution, and the eluate was concentrated to a small volume in vacuo at 50° C and made up to 10 ml. A portion of this was used for partition chromatography analysis.

Silica gel partition chromatography with a gradient elution technique was used for separation of organic acids in leaf extracts. The concentration of organic acids eluted was determined by titration with standard alkali. Silica gel was prepared from reagent grade sodium metasilicate essentially as described by Neish (10). However, to obtain sufficiently high flow rates on the column it was necessary to remove the finer particles by suspending the material repeatedly in water and decanting the upper liquid after the coarse material had settled. In this experiment the final pH of the washings was about 8.0.

The silica gel column was contained in a glass tube 25 cm long and 1.2 cm internal diameter. Four grams of dry silica gel were mixed with 3.5 ml of 1.0 N H_2SO_4 and slurried with 20 ml of $CHCl_3$. The slurry of silica gel was poured in gradually and transferred to the chromatographic tube in five or six portions so that about a four centimeter section of the bed was formed at one time. The gel was packed by inserting a circle of filter paper, pressed with glass tubing and adjusted to a final height of 18 cm.

The solvent mixtures employed were 2% and 50% (v/v) reagent grade tert-amyl alcohol in $CHCl_3$ (afterwards referred to as CA2 & CA50, resp.) The $CHCl_3$ was washed with distilled water to remove ethanol. The mixtures of CA2 and CA50 were equilibrated with 0.1 N H_2SO_4 and passed through filter paper to remove suspended water droplets.

Two milliliters of the sample solution were made alkaline to thymol blue and evaporated to dryness on a boiling water bath. The residue was cooled and dissolved in 1.5 N H_2SO_4 (about 1.3 ml) to adjust the pH to 1 to 2 with pH paper; 0.7 g of silica gel was mixed with the sample to give a free-floating powder. This was transferred to the top of the column, washed down and slurried with a small volume (5 ml) of $CHCl_3$ and packed under a filter paper disk. The tube was then filled with solvent mixture CA2.

The simple arrangement used for gradient elution consisted of two reservoirs. The recipient was a glass bottle of 250 ml capacity. The top of this bottle was connected to a 500 ml separatory funnel by a rubber cork. The recipient bottle contained 235 ml of CA2 and the funnel contained 450 ml of CA50. The column was fed from the recipient reservoir, which was stirred magnetically. Such a system delivered solvent continuously changing in concentration. By mounting the reservoirs about 80 cm above the column sufficient pressure was obtained to give a flow rate of approximately 18 ml/hour. The effluent was collected in 3.5 ml fractions by a drop counter fraction collector (fig 1).

The 3.5 ml effluent collected in each fraction was transferred to a 24 ml Ehrlenmeyer flask. Five milliliters of boiled distilled water were added and

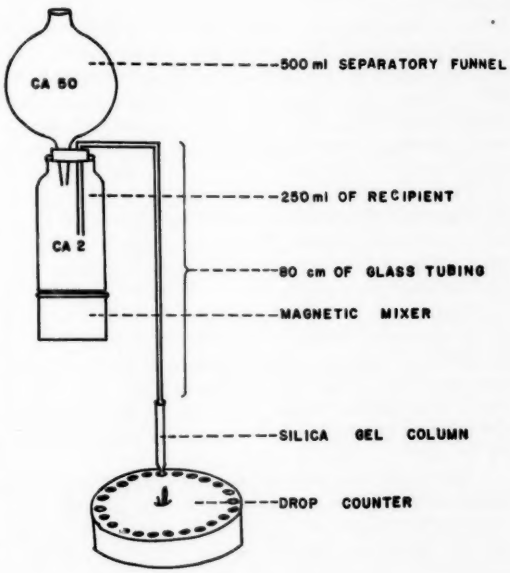

Figure 1. Gradient elution system

FIG. 1. Arrangement of apparatus for separation of organic acids on a silica gel column using gradient elution.

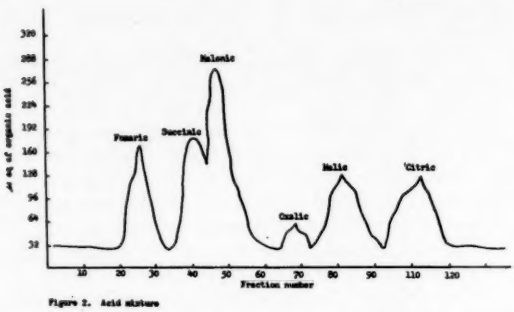

FIG. 2. Organic acid separation of a known mixture. Acids were separated using gradient elution of a silica gel column.

the sample was titrated with 0.01 N NaOH using thymol blue as indicator.

Results obtained from the separation of a synthetic mixture of acids are shown in figure 2. The titre for each fraction was plotted against the fraction number. This method gave good separation with well-defined peaks of fumaric, malic, and citric acids. Some overlapping of succinic and malonic acids occurred under these conditions. Better resolution was obtained if the recipient reservoir had a 300 ml capacity. In the experiments, overlapping of malonic and succinic acids occurred but it was often possible to separate them. Recovery of individual acids exceeded 95 %.

After titration of each fraction, the fractions within the same peak were combined. The water layer was separated and concentrated to about one milliliter. This solution was subjected to qualitative descenting paper chromatography. The general techniques followed were those of Buch, Montgomery, and Porter. (3)

The acid test solution and known acid solutions were spotted on Whatman no. 1 filter paper (45 × 19 cm) using a micro-fine pipette prepared for this purpose. The spot diameters were approximately one centimeter.

The solvent mixture employed was 1 penthanol and 5 M aqueous formic acid, prepared at least 3 hours before by combining equal volumes of the two components. The filter paper was equilibrated for 3 hours and then developed with the organic phase of the solvent system until the solvent front had moved 35 to 40 cm.

After development of the chromatograms, the sheets were removed from the tank, the solvent front was marked, and the solvents were removed by hanging the paper at room temperature for more than 2 hours in a stream of air in a chamber.

The spot locations were identified by spraying the sheet with bromophenol blue or with ammoniacal silver nitrate solution. The acidic spots could be seen immediately after spraying with the former reagent.

The content of iron was determined in the leaves of chlorotic and normal plants by Saywell's colorimetric *o*-phenanthroline method (12). One gram of leaves was used throughout the experiments (tables I, II, & III). Iron was not determined in the Mn treatments due to loss of samples.

Organic acid determinations from each plant sample were replicated three times. Variation was less than 5 %. The reported values are means of the three individual values. For all treatments except high phosphorous, organic acids were determined from two or more separate experiments in which the treatments were identical or similar. These organic acid determinations from different experiments of the same treatments are noted as sample A, B, or C.

RESULTS

IRON DEFICIENCY CHLOROSIS. Organic acid analyses of the iron-deficiency experiments are shown in table I. The leaves in sample A contained more total organic acids than in sample B, which was probably related to the difference in age of plants. Normal plants contained greater total organic acid concentrations than chlorotic plants, which was a reflection of the higher concentration in the malonate and succinate fractions. Only the citrate fraction showed greater accumulation in the chlorotic than in the normal plants. Iron content of the chlorotic leaves was slightly lower than that of the normal leaves in both samples A and B.

BICARBONATE INDUCED CHLOROSIS. As shown in table II, organic acid concentration was greater in chlorotic samples than in the normal. In chlorotic PI soybean tissue, malonate and citrate accumulated, whereas in chlorotic tissue of HA soybean, malate accumulated in addition to citrate and malonate. Total organic acid differences between chlorotic and normal tissues were greater in the HA experiment than in the PI experiment.

HIGH PHOSPHOROUS-INDUCED CHLOROSIS. The relationship between phosphorous-induced chlorosis and organic acid composition of the leaves is shown in table III. As noted in bicarbonate-induced chlorosis the total organic acid concentration was higher in chlorotic plants than in normal plants. Higher concentrations of organic acids were found in the chlorotic plants in the fumarate, malate, and citrate fractions. Citrate was the predominant acid in chlorotic and normal leaves and accounted for most of the increase under chlorotic conditions.

HIGH MANGANESE-INDUCED CHLOROSIS. Comparison of the organic acid composition of chlorotic and normal leaves is shown in table IV. The results are similar to those observed with iron deficient treatments. Little difference was noted in total organic acid concentrations between chlorotic and normal leaf tissue. The succinate-malonate fraction was greater in normal than chlorotic tissue. Only the citrate concentration increased under chlorotic conditions.

Discussion

Less iron was found in the chlorotic leaves than leaves from comparable control plants. It was also evident that increases in the citric acid content occurred in chlorotic tissue regardless of how chlorosis was induced. Citric acid existed in largest amounts in foliage that was most markedly chlorotic. Similar findings were observed earlier by Iljin (6, 7, 8) and Schander (13, 14, 15) in lime-induced chlorotic tissue. Other acids isolated were fumarate, succinate, malonate, and malate. These observations indicated the presence of four of the Kreb's cycle acids and suggested the existence of the Kreb's cycle oxidation scheme in PI and HA soybean leaves. The accumulation of citric acid in chlorotic leaves may be due to a block in the pathway of amino acid and protein synthesis from organic acids, or abnormal operation of the Kreb's cycle.

Wadleigh and Brown (16) and Lindsey and Thorne (9) concluded that lime-induced chlorosis symptoms were similar to iron deficiency chlorosis in many respects, and suggested that bicarbonate-induced chlorosis symptoms were analogous to lime-induced chlorosis symptoms. The present data indicated that iron deficiency-induced chlorosis was not synonymous with bicarbonate or lime-induced chlorosis with respect to organic acid changes observed in chlorotic tissue.

Striking differences in malonate accumulation could be found in chlorotic tissues in comparison between iron deficiency chlorosis and bicarbonate-induced chlorosis. In bicarbonate-induced chlorotic leaves the malonic acid concentration was much higher than in control leaves. Conversely, in chlorotic plants growing in the low iron solutions the malonic acid concentration was higher in the control plants. Little difference could be found in total organic acid concentration between chlorotic and normal tissue in the iron deficiency experiments, but marked differences were observed between chlorotic and normal plants in total organic acid concentration in the bicarbonate experiments. The difference in pH values of the nutrient solutions of the two treatments (5.5 for iron deficiency & 7.8 for bicarbonate) may account for some of the differences observed between treatments. Each treatment was compared to a control where pH was not a variable which indicated an effect of bicarbonate and minus iron on organic acid metabolism independent of pH. High phosphorous-induced chlorosis was similar to bicarbonate-induced chlorosis with respect to organic acid composition of chlorotic leaves. This observation is interesting in view of the experiments in which Brown et al (2) suggested that the effect of HCO_3 on chlorosis was indirect and linked to its effect on phosphorous solubility. Brown (1) suggested further that the bicarbonate ion was a factor contributing to the development of iron-deficiency chlorosis through its indirect action on entry and activity of iron. Though it is not clear whether malonate metabolism is related to the development of bicarbonate chlorosis, the present data indicate that bicarbonate-induced chlorosis or high phosphorous-induced chlorosis was not similar to iron deficiency chlorosis in respect to malonate metabolism and total organic acid concentration.

In the high manganese-induced chlorosis experiments, malonate concentration was much higher in control leaves than in chlorotic leaves, but there was no difference in total organic acid concentration. The organic acid composition of chlorotic leaves from the iron deficiency and high manganese experiments shows a similar pattern. Direct comparison cannot be made since the nutrient solutions of the two treatments differed in mineral concentration. It is postulated, however, that high manganese in the nutrient solution competed with iron absorption and upset the metabolism in a way similar to that in iron deficiency experiments.

Summary

Experiments were conducted on iron chlorosis in higher plants as related to organic acid content. The *Glycine max* variety PI–54619–5–1 (PI) soybean was grown in growth chambers where light intensity, day length, and temperature were controlled.

Four types of iron chlorosis were induced: A, iron deficiency induced chlorosis; B, bicarbonate induced chlorosis; C, high phosphorus induced chlorosis; D, high manganese induced chlorosis.

Soybeans were harvested and 25 g of leaf tissues were analyzed qualitatively and quantitatively for organic acid by silica gel partition chromatography.

Chlorotic leaves contained less iron than control leaves in three treatments.

Significant increases in citric acid content were found in the leaves of chlorotic plants when compared to the leaves of control plants regardless of how chlorosis was induced. The leaves of plants grown in iron-deficient nutrient mediums or in solutions containing high phosphorous concentrations developed chlorosis and contained much less malonic acid, when compared to control plants for each treatment. When chlorosis was induced in plants by bicarbonate or high manganese treatments, the malonic acid concentration was markedly higher in leaves from the chlorotic plants than from comparable control plants.

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DISTRIBUTION OF BORON IN LEAVES[1]

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The boron content of plant tissues has been investigated extensively by Eaton (2). From his data it can be readily ascertained that when the boron supply of the plant is adequate or excessive the leaves contain consistently higher concentrations of boron than do other plant parts. For instance, he has shown that the roots of sugar beets showing symptoms of boron excess contained 28 to 52 ppm[3] of boron while the leaves contained 495 to 1,008 ppm. The foliage of grapes showing neither deficiency nor toxicity symptoms was found to contain 250 to 267 ppm while the stem contained only 50 ppm. Thus, it has been shown that boron tends to collect in the leaves which are, as well, the endpoint for the transpiration stream of the plant. Furthermore, Eaton (2) and Oertli (4) have both shown that the concentration of boron is highest in the marginal, necrotic areas of the lemon leaf from a plant to which excess boron was applied and lowest at the base of the midvein. Such a situation can be explained by assuming that boron is passively carried within the plant in the transpiration stream.

The objective of the authors in performing the experiments reported in this paper was to further investigate this matter of the passive transport of boron in the transpiration stream by studying the distribution of boron in some leaves with simpler venation than that of lemon leaves.

MATERIALS & METHODS

For the most part materials and methods will be described briefly with the report of the results of each experiment. However, the method of boron analysis is common to all experiments and is, therefore, described here.

Plant tissues after being dried and weighed were ashed in cylindrical 20 ml crucibles. Then, after cooling, 0.5 ml of 0.1 N HCl and 2.0 ml of cucurminoxalic acid reagent (1) were added to each sample. After drying at 55° C the residue was dissolved in 10 ml of ethyl alcohol. Finally, the optical density of the solution was determined at 5,500 A with the aid of a Beckman spectrophotometer (Model B). Possible interference by a calcium oxalate precipitate was

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[3] Unless otherwise stated, concentrations are expressed on a dry weight basis.

avoided by allowing the precipitate to settle out before decanting the liquid into the cuvettes.

The sensitivity of the method was such that the difference between 0.01 and 0.02 micrograms of boron could be detected. Plant samples were of such a size that they contained 0.1 to 2.0 micrograms of boron.

All plants used in this work were grown in a 16° C greenhouse in a soil mixture of half sand and half sphagnum peat. All plants received 0.3 ppm boron in the regular liquid fertilization program. Some plants, as will be noted, received additional boron.

Results & Discussion

If boron is passively carried in the transpiration stream, one might reasonably expect the boron concentration to be higher near the tip of a parallel veined leaf than at the base in view of the facts that the transpiration stream is unidirectional and that boron movement from the leaf is very limited (3).

Such an increase in boron concentration from base to tip was shown to exist in the leaves of Easter lilies (*Lilium longiflorum* Thunb. var. Ace) (fig 1). For this experiment matured leaves showing no excess boron injury, but located immediately above the lower zone of boron-injured leaves having tip necrosis, were harvested from plants which had received 5 ppm boron in the irrigation water for 3 weeks previous to harvest. The leaves were sliced transversely in 2 mm sections which were analyzed for their boron content. Near the tip the boron concentration was found to be almost 30 times that found at the base of the leaf (fig 1). The pattern and boron concentration were the same for the three leaves analyzed except that the concentration at the maximum varied between 2,500 ppm and 3,800 ppm, which might well be expected considering the rapid change in boron concentration on either side of the location showing maximum concentration.

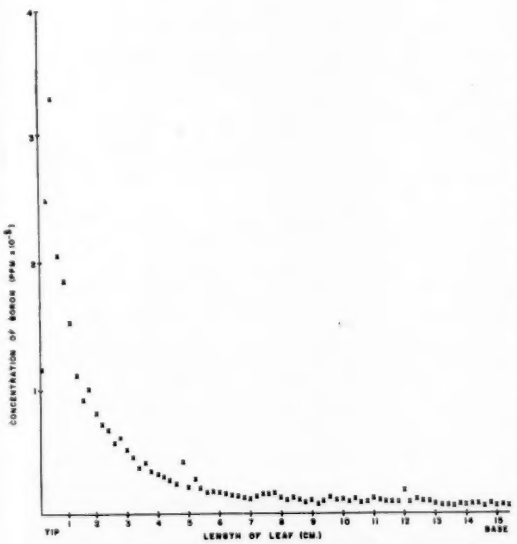

Fig. 1. The concentration of boron in 2 mm transverse sections of an Easter lily leaf indicating the regular increase in concentration from the base to near the tip. Data also indicate the accuracy of the method of analysis.

This pattern of boron distribution was also found in the leaves from plants which had received a lower concentration of 0.3 ppm boron in the irrigation water although the concentrations found were lower throughout the leaf (fig 2).

If this pattern of distribution in the lily leaf is the result of boron being passively carried in the transpiration stream, then the magnitude of boron concentration at any point on the leaf should be accounted for by considering two physical properties of the system—the evaporation of water and the largely irreversible, uniform flow of water from the base to the tip of the leaf. As water evaporates from the transpiration stream as it passes through the leaf, the solutes, including boron, would become more concentrated in the remaining solution. Since, when half the water remained, the concentration of boron would be doubled, and when 25 % of the water remained, the concentration of boron would be quadrupled, etc., then the following equation may be written to indicate the concentration of boron in an aliquot of the transpiration stream as water is lost from it by transpiration: $C_a = CV/V_a$ where C_a is the concentration of a solute (boron) after water has evaporated to leave the volume V_a. C is the original concentration of the solute in the aliquot having the original volume V.

Provided the leaf transpires equally over all its surface, and rather crude experiments on the rate of evaporation from various sections of the leaf indicate that this is possible, then the volume of an aliquot of the transpiration stream is proportional to the per centage of the leaf area not traversed, since 100 % of the water in the aliquot will be lost by the time the aliquot reaches the leaf tip. Leaf area may then be substituted for volume in the preceding equation and the following formula derived: $C_a = CA/A_a$ where C_a is the concentration of the solute (boron) at the line where there is A_a area from there to the tip of the leaf. C is the concentration of the solute in the transpiration stream as it enters the leaf, and A is the total leaf area.

Applying the above formula, a good fit is obtained between calculated (for C = 63) and found concentrations (fig 3). Such a good fit substantially supports the hypothesis that boron, at least when present in adequate amounts, is passively carried in the transpiration stream. However, contrary to expectations, the concentration of boron at the tip is lower than that 8 to 10 mm from the tip. The solution guttated from the leaf tip on some nights may cause this situation since that part of it reabsorbed the following day should result in an increase in boron from the tip to-

ward the base. As evidence supporting this explanation the authors have found that such an exudate from corn leaves normally contains considerable boron.

To further test the hypothesis that boron is passively carried in the transpiration stream, experiments were designed to find if the boron in the living leaf is readily soluble in water. Eaton (2) has shown that most of the boron contained in the leaf tissue is removed when the juice is squeezed from the leaves in a press. From this it may reasonably be assumed that boron in the leaves is water soluble, which would be necessary if the passive transport hypothesis is valid. However, additional evidence to that of Eaton seemed desirable since the solubilities of substances within cells and tissues are hardly conclusively established by the solubility in plant sap expressed under a pressure of 2,400 pounds per square inch, and so two experiments were performed to this end.

In the first experiment, lily leaves, each harvested from similarly treated plants and from similar loca-

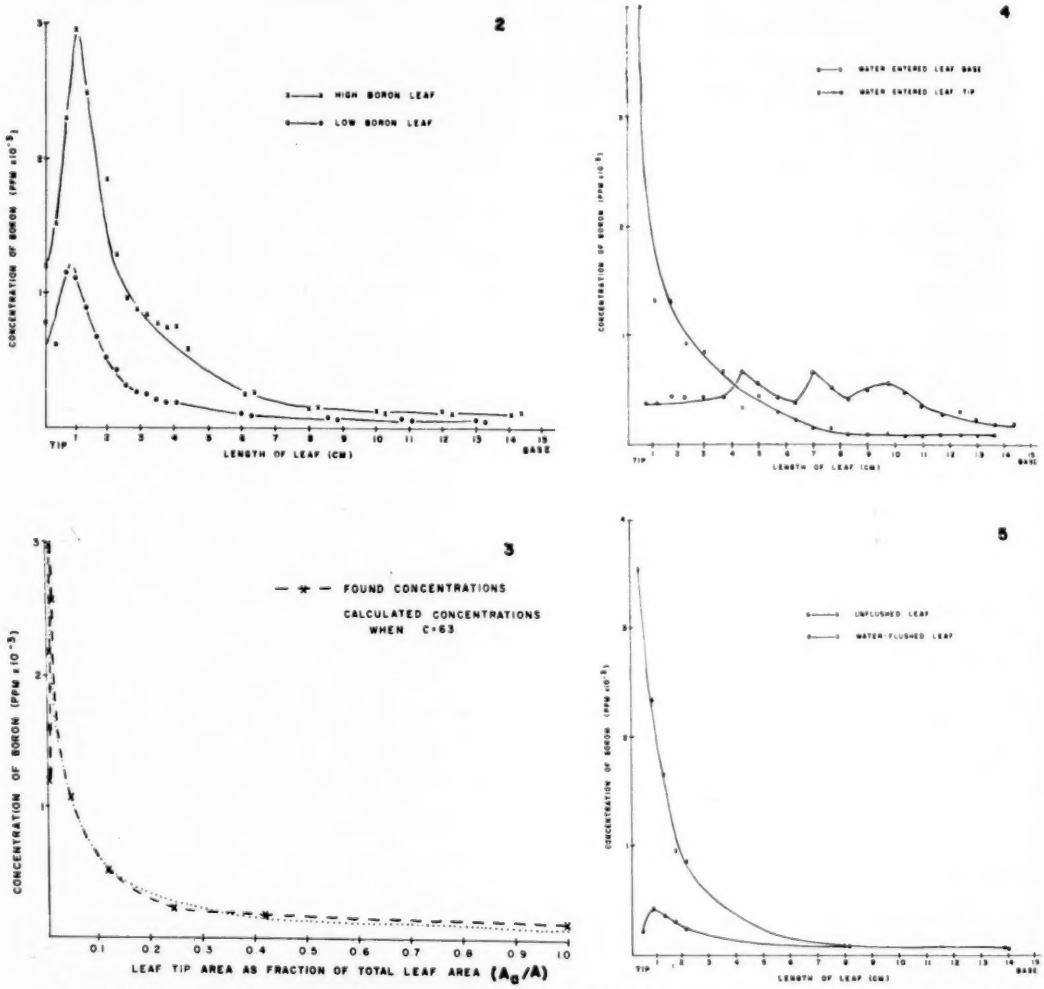

FIG. 2. The concentration pattern of boron in a leaf of an Easter lily plant which received 5 ppm in the irrigation solution and of a leaf from a plant which received 0.3 ppm.

FIG. 3. Calculated boron concentrations for an Easter lily leaf compared with the found values. For the formula used see the text.

FIG. 4. Concentrations of boron at various locations in an Easter lily leaf allowed to take up distilled water from the base compared with those of a leaf allowed to take up distilled water from the tip. Both leaves had concentration patterns similar to that in figure 1.

FIG. 5. Comparison of concentrations of boron in an untreated leaf of rubrum lily with those in a leaf through which distilled water was forced.

tions and presumably having an original boron concentration pattern similar to that of the leaf in figure 1, were removed from the plant, placed in vials of distilled water, and then placed under flood lights so that they would transpire rapidly. Blank vials were included so that evaporation could be subtracted from total loss of vial solution, the difference being taken as the amount of water entering the leaves. Since all vials were protected from the light with aluminum foil the evaporation loss was small (0.87 ml). The base of each of four leaves was submerged about four centimeters in the distilled water. The tips, after removal of the tipmost centimeter, of each of four other leaves were submerged the same amount. With these latter leaves the transpiration stream was thereby made to flow from tip to base. After 25 hours the leaves, seemingly uninjured, were removed and cut transversely into 3 mm sections for each of which the boron concentration was ascertained. The data, graphically presented for a typical leaf of each treatment in figure 4, indicate that the boron in the leaf was rather readily moved by the entering water and hence could not be considered as fixed by the tissues.

In another effort to ascertain the mobility of boron in leaf tissue an attempt was made to reduce the boron concentration in a leaf by forcing boron-free water through it. The basal section of a stem-piece of rubrum lily (*Lilium speciosum* Thunb. var. rubrum) on which a leaf was allowed to remain was passed through a one-holed rubber stopper which was placed in the top of a sidearm flask so that the cut end of the stem was under water. Then the apical cut of the stem was covered with rubber tubing which was clamped so that water could not escape. The apparatus containing the stem piece was placed under a bell jar; 2 mm of the tip of the leaf were removed; and by means of compressed air, introduced through the sidearm, the pressure in the flask was increased by 40 cm of mercury. As a result of this treatment liquid exuded from the cut end of the leaf and was allowed to drop into a crucible. The experiment was conducted twice, several samples being collected each time.

Analysis of the exudate revealed that boron was being flushed from the leaf in considerable quantity. Furthermore, the first exudate was higher in boron than later samples. Analysis of the leaf after treatment (fig 5) revealed that the boron forced from the leaf was primarily from the tip section where the concentration was markedly lower than that found in the same portion of that leaf immediately above the treated leaf. It is assumed that the boron distribution in the untreated leaf immediately above the water-flushed leaf substantially represented the original distribution in the water-flushed leaf. The results of this experiment confirm the results of the reverse flow experiment with Easter lily leaves. Both experiments indicate that the boron in the leaf is not fixed by the tissue and hence the experiments support the hypothesis that boron is carried passively in the transpiration stream of the leaf.

Conclusions & Implications

It is concluded from the data presented that the boron distribution in the leaf may be accounted for in the main by considering the movement of water into and from the leaf. Boron is more concentrated in those areas of the leaf which might be considered sinks for water in that the mass flow of the transpiration stream ends in these areas. For parallel veined leaves such as those of Easter lilies there is a single such sink for water at the end of the leaf. For those leaves with more complex venation, margins and even internal leaf areas may be such end points.

Since the movement of water into and from the leaf is, for the most part, considered to be a passive process, i.e., dependent upon physical characteristics of the system only, then boron distribution in the leaf may likewise be considered a passive process.

The above statements should be limited to indicate that they describe the boron distribution only when the plant is receiving adequate or excessive amounts of boron. It is probable that when boron is supplied in only small amounts the above explanation fails to be accurate since there is almost certainly some active cellular uptake or fixation of boron possibly by OH groups in cellulose and other cell constituents (3). This fixation would not be proportional to the concentration of boron in the transpiration stream. There are two indications of such a fixed increment of boron in the data presented. In the mathematical treatment of the data in figure 3 a still better fit could have been made between the found and calculated concentrations if it had been assumed that there was a fixed amount of boron of about 35 ppm in the tissue. This would mean amending the suggested formula by adding this constant or near constant. Less convincing that there is a fixed quantity of boron in the tissue is the fact that when boron-free water was passed through the leaves, the boron was not completely removed. However, since part of the process of establishing equilibrium is probably dependent upon diffusion of water and boron through solid tissue, a slow process, it is reasonable to assume that not enough time was allowed during the experiments for the boron to diffuse from these tissues. Hence, the data become undeterminable as to whether there is a fixed quantity of boron in the leaf.

Be that as it may, the data indicate conclusively that most of the boron in leaves of plants supplied sufficient boron, is water soluble and readily moved (figs 4 & 5). Still, it has been reported that when plants which have been supplied sufficient boron are changed from such a situation to one where they are not supplied boron their new tissues are deficient in boron (3). In view of the data presented here, this situation is only conceivable if the mobile boron in the older leaves cannot be translocated from the leaves, which, in turn, implies that boron is not carried from the leaf in the phloem and suggests that the phloem membranes are largely impermeable to boron.

Eaton (2) has hypothesized the formation of large molecular boron compounds which cannot pass through cell membranes to explain this situation but in view of the ease with which boron is moved in the leaf by water this seems unlikely.

The data have several other interesting implications. First of all it is almost certain, in view of the fact that high boron concentration and necrotic leaf tips in lilies occur at the same location, that the toxic effect of boron is local and direct in that the tip necrosis does not occur as a secondary effect resulting from injury to some other part or tissue of the plant.

Also of interest is the implication that if the leaf lives long enough it will show symptoms of boron toxicity even though the plant is supplied boron at levels ordinarily considered non-toxic. This follows since each aliquot of water entering the leaf brings with it boron which eventually accumulates at the end points of the transpiration stream and is not removed.

This last statement, that for most plants the boron is not removed from the leaf, must certainly be amended to consider the possibility that considerable boron can be removed in guttation water. Conditions which lead to a high loss of solution by guttation may then be considered a protective mechanism against boron injury in the leaf.

One further conclusion from the data is that when analyses are made for diagnosis of boron toxicity special consideration should be given to the tissue in the immediate area where there is necrosis or where necrosis would be expected to appear first. The analysis of other plant tissues, including other portions of the leaf, may be quite misleading.

Summary

The distribution pattern of boron in leaves of Easter lilies and rubrum lilies was found to be a hyperbolic increase from the leaf base to near the leaf tip. This, along with the finding that much of the boron in the leaves of these species was mobile, led the authors to the conclusions that:

I. Boron moves passively in the transpiration stream and that, in the main, the distribution of boron in the leaf may be accounted for by considering only a few physical parameters of the system.

II. The lack of transport of boron from the leaves is probably due to lack of transport by the phloem.

III. Injury to the leaf by excess boron is local and primary rather than secondary as a result of injury to some other plant part.

IV. Loss of guttation solution may be a protective mechanism against boron toxicity.

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EFFECT OF SOIL OXYGEN DEFICIT ON IRON NUTRITION OF ORANGE SEEDLINGS[1, 2]

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Iron deficiency chlorosis in plants is often associated with soil conditions that appear to restrict exchange of gases with the atmosphere, such as fine texture, lack of aggregation, excessive moisture, or discontinuity in the profile in the form of plowsole, hardpan, claypan, or stratified deposits. These conditions can cause CO_2 to accumulate in the soil and, particularly in calcareous soils, produce substantial concentrations of HCO_3 ions. Solution culture studies (1, 14) have shown that $NaHCO_3$ can cause a marked reduction in iron absorption by some kinds of plants. Thus has arisen the so-called bicarbonate hypothesis to explain the variable chlorosis of plants growing in calcareous soils.

The bicarbonate hypothesis represents a sound approach to the problem. The difficulty is that a causal relationship between bicarbonate ion and chlorosis in soil-grown plants has not been established. On the contrary, Reuther and Crawford (9, 10) increased concentrations of CO_2 in the root zone of young citrus trees growing in calcareous soils to levels of 20 to 25 % without inducing iron chlorosis. The present authors grew sweet orange seedlings in calcareous soil in the greenhouse and added 1 % of CO_2 to the soil atmosphere over a period of several months without causing chlorosis or reducing the iron concentration in the leaves (data unpublished).

Impeded exchange of gases between the soil and the atmosphere can result, in addition to the accumulation of CO_2, in reduction of oxygen concentration. The present study was undertaken to determine if oxygen deficit in the soil may bear a causal relation to iron chlorosis in plants.

MATERIALS & METHODS

Concentration of oxygen in the soil was controlled by regulating concentration in the atmosphere in contact with the soil surface. This was accomplished, using a soil of high porosity, by enclosing the space over the soil and introducing into it gas of controlled composition. The experiment was conducted in a greenhouse using 3-gal, glazed ceramic jars as containers.

The soil was a virgin sandy loam of granitic origin, pH 6.2, taken from an alluvial deposit in a hilly area of chaparral vegetation. It was pretreated with 0.1 % of vinyl acetate-maleic acid copolymer (VAMA) to give maximum porosity.

Seedlings of sour orange (*Citrus aurantium* Linn. var. Standard) were transplanted at age 4 months, two plants per jar, and four replicate jars per treatment. Jars were arranged on a single bench in a randomized block design. A tensiometer was installed in each jar to provide a continuous measure of soil moisture. Preliminary tests showed that soil aeration was completely adequate for plant growth when the watering program permitted a maximum suction value of 0.1 bar. In order to avoid the possibility of operating on the borderline of deficient gas exchange, a value of 0.2 bar was adopted for the present experiment. The watering procedure was to add 300 ml of water (purified by ion exchange) to each jar when the suction value reached 0.2 bar, which established the limits of soil water concentration in the range 8 to 11 %. Remaining pore space exceeded 30 %.

Nitrogen was supplied to the plants in the form of a solution of mixed nitrates of calcium and magnesium, added at the start of the experiment and once during the experiment in quantity sufficient to give 50 ppm N in the soil solution at each addition.

After the plants were well established, each jar was covered with aluminum foil which was fitted closely around plant stems and sealed to the rim of the jar, to the tensiometer stem, and to the glass tube inserted for application of water. The desired gas mixture was injected continuously into this space at a rate between 50 and 100 cc per minute. Samples of soil gas were obtained by the equilibrium method of Taylor and Abrahams (11) by connecting a bottle to a glass tube 10 mm ID that was installed through the drain hole near the bottom of the jar so that the inner end was close to the center of the soil mass. Oxygen was measured polarographically, using a rotating platinum electrode as described by Kolthoff and Lingane (6), calibrated with known gas mixtures. This method of measurement gave the same results as the volumetric absorption method but was considerably more rapid.

Occasional measurements of CO_2 concentrations in the soil were made by the volumetric absorption method. In no case did the measured values reach 1 %.

Soil treatments consisted of untreated controls and four levels of $CaCO_3$: 0.5 %, 1 %, 2 %, and 3 %.

[1] Received December 22, 1960.

[2] Citrus Experiment Station Paper no. 1278.

Duplicate sets received different oxygen concentrations, the first receiving unaltered compressed air and the second a mixture of nitrogen and air to give oxygen concentrations in the soil in the range 3 to 5 %.

Three weeks after application of differential oxygen treatments, small string tags were applied to the growing tips to identify leaves of uniform age for analysis. At the time of harvest (2 months later) all fully expanded leaves above the tags were harvested separately and designated as standard leaves. All leaves and stems were washed individually with soap prior to being dried and ground for analysis.

As the soil was removed from each jar, two soil samples were taken as quickly as possible. To the first, about 25 g, pure water was added in quantity just sufficient to make a saturated paste and pH was measured with a glass electrode. The second, about 2 kg, was tamped into a metal cylinder and free water added on top for displacement of soil solution under pressure. Bicarbonate ion concentrations in the expressed solutions were determined by direct titration with standardized HCl.

Chemical analyses of plant materials were done by methods previously described (13), plus zinc and copper as outlined by Johnson and Ulrich (5), and magnesium by titration with Na_2EDTA following removal of calcium by precipitation as oxalate.

Analysis of variance was used to estimate significance of differences between means.

Results

Soil pH values and concentrations of bicarbonate ions in the soil solution are given in table I.

Plant weights and tissue analyses are reported in table II. Data for the cultures that received $CaCO_3$ have been combined under the heading "$+CaCO_3$" because they did not show any consistent effect of increasing concentration of $CaCO_3$.

Table I

pH Values of Soils & Bicarbonate Ion Concentrations in Soil Solutions at End of Experiment

% $CaCO_3$	pH of Soil		Mean conc HCO_3^- me/l
	Range	Mean	
	High oxygen level		
0	6.1–6.4	6.25	2.2
0.5	7.3–7.6	7.52	5.3
1	7.5–7.6	7.52	3.8
2	7.3–7.6	7.55	2.8
3	7.4–7.7	7.52	4.8
	Low oxygen level		
0	6.1–6.2	6.18	1.5
0.5	7.2–7.5	7.35	2.4
1	7.3–7.8	7.50	3.4
2	7.3–7.5	7.40	2.8
3	7.2–7.6	7.42	2.9

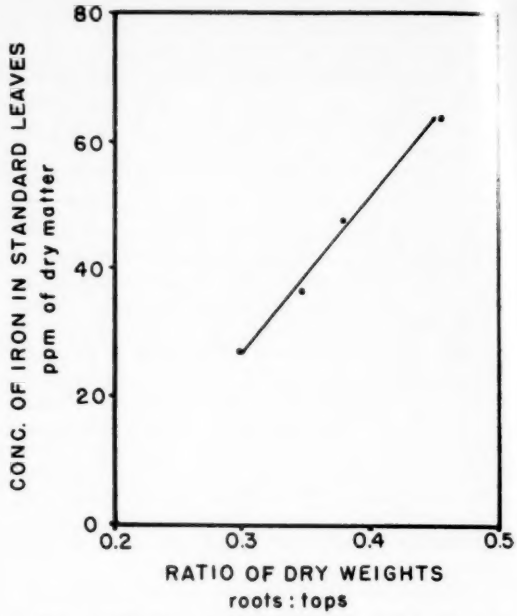

Fig. 1. Relationship between iron concentration in standard leaves and root: top ratio of plants.

Oxygen deficit resulted in significant reduction of plant growth, of chlorophyll and iron concentrations in the leaves, and of total iron content of leaves and stems. Presence in the soil of $CaCO_3$ likewise resulted in decreased concentration of iron in the leaves as well as decreased total iron in leaves and stems. Its effect on plant growth and on chlorophyll concentration in leaves was not significant.

These effects were evident from visual inspection of the plants; production of new leaves was noticeably retarded 3 weeks after application of the low oxygen treatment; at the conclusion of the experiment, new leaves in the $+CaCO_3$, low oxygen series bore typical iron chlorosis patterns varying from moderate to severe intensity.

Comparisons of nutrient elements other than iron are limited by lack of sufficient leaf material in the low oxygen treatment. A few comparisons were made, where enough material was available, but may be somewhat biased because they represent the larger plants. Manganese appears to have been affected in a manner comparable to iron. There is also a suggestion that low oxygen in the presence of $CaCO_3$ resulted in reduced copper concentration. Differences for Zn, Mg, and K were not significant.

A substantial correlation existed ($r = 0.76$) between the bicarbonate ion concentrations and root weights in individual jars of the $+CaCO_3$ series. This is interpreted to mean that HCO_3 ion concentration was controlled primarily by the amount of CO_2 evolved by the roots.

Discussion

Deficiencies of iron and manganese in plants grown in calcareous soils are commonly attributed to pH effects on solubility of these elements (8). Explanation is still needed for the fact that chlorosis of susceptible plants does not always occur in calcareous soils. Solid phase carbonates exert such a strong buffering action that variations in pH would seem to be inadequate to account for the variable occurrence of chlorosis.

Results of the present experiment indicate that oxygen deficit in soils may be a contributing cause of iron (& manganese) deficiency in the field. Absorption of both elements was depressed either by adding $CaCO_3$ to the soil or by reducing the oxygen concentration in the soil; neither factor, by itself, caused visible chlorosis but the combined effects were additive and caused distinct chlorosis. If subsequent studies show that oxygen deficit is sometimes a factor in producing chlorosis in the field, then variations in soil oxygen level may account for some of the observed differences in severity of chlorosis.

The mechanism for these effects is not immediately apparent. It was not operative in the solution culture study reported by Lindsay and Thorne (7) in which oxygen concentrations as low as 2.5 % in the aeration stream failed to depress iron absorption by bean plants. This would suggest that the mechanism may be operative only in soils. Furthermore, the fact that HCO_3 ion concentrations were lower in the low oxygen cultures than in the high oxygen cultures indicates that a CO_2-HCO_3 ion mechanism was not involved in the responses to oxygen level.

One possible explanation of the oxygen effect is that metabolic ion absorption was depressed at the low level of oxygen. This seems doubtful, however, in view of the following considerations:

A, If such a mechanism exists, it should have depressed iron absorption in the studies of Lindsay and Thorne (7); B, Studies by Vlamis and Davis (12) using potassium and bromide ions indicate that

Table II

Effects of Added $CaCO_3$ & Reduced Oxygen Tension in Soil on Plant Weights, Total Iron in Tops, & Chemical Composition of Standard Leaves

	Soil treatments				Statistical significance for effect of:	
	High oxygen*		Low oxygen*			
	$-CaCO_3$	$+CaCO_3$**	$-CaCO_3$	$+CaCO_3$	O_2	$CaCO_3$***
			Mean weight per jar			
Dry wt of tops, g	27.9	26.3	22.9	20.5	++	NS
Dry wt of roots, g	12.8	9.8	7.9	5.7	+++	+
Dry wt of entire plants, g†	40.7	37.0	30.50	26.4	+++	NS
Dry wt of standard leaves, g	6.45	6.77	3.47	2.22	++	NS
Wt. of Fe in tops, mg	1.62	1.16	0.82	0.64	+++	++
Dry wt ratio of roots: tops	0.457	0.378	0.348	0.296	+++	++
			Chemical composition of standard leaves††			
Fe, ppm	64	48	37	27	+++	+++
Mn, ppm	20.2	15.2	...	...	...	+++
Zn, ppm	19.1	17.1	...	...	...	NS
Cu, ppm	8.9	7.2	...	...	...	NS
Mg, %	0.31	0.30	...	...	...	NS
K, %	0.87	0.90	...	...	...	NS
Mn, ppm (8)‡	...	15.6	...	6.6	+++	...
Zn, ppm (8)‡	...	17.4	...	16.9	NS	...
Cu, ppm (5)‡	...	9.1	...	5.4	+	...
Mg, % (5)‡	...	0.29	...	0.28	NS	...
K, % (5)‡	...	1.14	...	0.98	NS	...
Chlorophyll, mg/g	3.60	3.68	2.70	2.45	+++	NS

* High oxygen treatment: oxygen concentrations from 18 to 21 %; low oxygen treatment: oxygen concentrations from 2.0 to 6.2 %, mean 3.5 %.

** Minus $CaCO_3$ indicates original soil, pH 6.2; plus $CaCO_3$ values are means of all soils that received $CaCO_3$.

*** Symbols +, ++, +++ indicate statistical significance at the 0.05, 0.01, and 0.001 levels of probability, respectively. NS indicates difference found is not shown to be significant.

† Sum of wood and leaves. The woody parts were weighed before separation of roots and stem.

†† Mean concentration on dry weight basis.

‡ Total weight of standard leaves was not sufficient in all plants to permit complete chemical analyses, particularly at the low oxygen level. In such cases, a limited number (indicated in parentheses) of single comparisons were possible.

oxygen becomes an important limiting factor in ion absorption only at concentrations below 2.5 %; C, In the present experiment the roots continued to grow, at a reduced rate, under low oxygen conditions; D, Leaf concentrations of Mg, K, P, and Zn were not significantly different from those in the control plants.

Another aspect worthy of some attention is the effect of oxygen on root growth. Gingrich and Russell (4) found that elongation of corn roots was retarded when subjected to soil oxygen concentrations below 10 % by volume. They did not show whether this effect was preferential for roots or simply part of an overall reduction in plant growth. In the present experiment this distinction was made by calculating the root: top ratios of dry weights, (table II). The ratio was found to be decreased (i.e., there was a preferential reduction in root growth) by the presence of $CaCO_3$ or by oxygen deficit in the soil and, as in the case of iron concentrations, the two effects were additive. By plotting leaf iron concentrations against root: top ratios, a close approximation of a straight line was obtained (fig 1).

The implication of this graph is either that iron absorption from this soil was a function of root: top ratio or that root: top ratio was a function of iron nutrition. Available data do not permit a distinction. If the first interpretation is correct it means that iron absorption is a strong function of root development. This is supported by observations by Eaton (3), and Chapman (2) that citrus plants can absorb iron from particles of magnetite (Fe_3O_4) in close contact with roots. It apparently deserves further investigation as a possible mechanism by which various factors, such as soil moisture, soil temperature, light intensity, and soil-borne pathogens influence iron nutrition of plants.

Summary

Sweet orange seedlings were grown in greenhouse pot cultures using an acid (pH 6.2) sandy loam soil with and without added $CaCO_3$. Duplicate series were subjected to differential soil oxygen levels (approximately 20 % vs. 3.5 %) during the last 2 months of growth.

The lower oxygen level proved to be deficient; plant growth was visibly retarded within 3 weeks, both in the acid and calcareous soils. Plants in calcareous soils at low oxygen level developed strong typical iron chlorosis whereas all other plants were normal in appearance.

Leaf analyses showed significant reduction in concentrations of iron and manganese resulting from both low oxygen and added $CaCO_3$. The effects of these two factors were additive in their adverse effects on iron and manganese nutrition. It was also found that leaf iron concentrations were proportional to relative size of the root system, which led to the suggestion that the relation of root growth to iron nutrition be investigated further.

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TRANSPIRATION STREAM & ASCENSION OF CALCIUM [1, 2]

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INTRODUCTION

Since Cline's report (5) of a very slow equilibration in the leaves of tritiated water absorbed by the roots of bean plants, no resolution has been made between his expressed alternative views that this was due A, to failure of tissue water to exchange with the incoming tritium-labeled water, or B, to a large isotope effect. During the preparation of the present manuscript Vartapetyan and Kursanov (14), using H_2O^{18}-enriched water in the nutrient solution, have also reported a slow equilibration of this tracer in the leaves (& roots) of the bean plant. In addition, they grew sunflower plants from seed with H_2O^{18}-enriched water and observed almost complete equilibration in all parts of the plant and thereby showed that the failure to equilibrate was due to failure of mixing, and that there remained the possibility of only an insignificant isotope effect. Even this was explainable by unlabeled water brought into the experiment in the seed, or to the formation of metabolic water in which there was a slight isotope effect.

Our report confirms the slow equilibration in leaves of the labeled water delivered in the transpiration stream, and in addition shows the progress of the labeled water through the stem and into the leaves in a way which discloses the path and manner of movement of the transpiration stream. The Ca^{45} isotope was used with the tracer water in order to compare the ascent of a mineral marker. Calcium was selected as it is not subject to a significant redistribution from leaves and could be expected to reside in the leaves into which it was delivered (4). The degree of dependence between the movement of a mineral component and the movement of the transpiration stream has never been satisfactorily investigated. The traditional concept is that mineral nutrients move upward in the xylem vessels in conjunction with the transpiration stream; once started the water and minerals move together and, with the exception of those salts removed by absorption along the way, reach their final destination in the leaves (6). The data obtained in the present study indicate that neither the water nor the calcium ascends exclusively in the vessels, but the stem as a whole functions, to some extent, as a pathway for ascent. As a result, a substantial portion of the ascending calcium moves by exchange on biocolloids rather than by mass movement in the vessels.

METHODS

Bean plants (*Phaseolus vulgaris* L. var. Red Kidney) were grown in a half-strength Hoagland solution, with micronutrients and aeration, to a stage where the third trifoliate leaf was about half expanded. The growth conditions were: temperature 23± 1° C, light 1,000 to 1,200 ft-c (fluorescent) on a 12-hour photoperiod, relative humidity 60 ± 5%. Two series of four plants each were then placed in a treatment chamber maintained under conditions similar to those above and wherein the water in the nutrient solution was labeled with THO and the calcium with Ca^{45}. In the first series tritium, as THO, was present at 0.0207 μc/ml and Ca^{45} at 0.167 μc/ml; in the second series THO was present at 0.0196 μc/ml and Ca^{45} 0.0167 μc/ml. The treatment chamber was designed to exhaust the air flowing over the leaves in order to remove any THO vapor transpired.

Plants were removed from the tracer solutions in the treatment chamber at 0.75-, 1.5-, 3-, and 6-hour intervals in the first series and 6-, 24-, 48-, and 72-hour intervals in the second. As each plant was removed, the hypocotyl and root were cut from the stem at the cotyledonary node (which was 2± ⅛ in above the root) and discarded. The top was divided into the following sections: primary leaves, first, second, and third trifoliate leaves, five basal stem sections of 1-inch length, each divided into bark and wood, two sections above these (undivided), and top (the remainder). Bark, as herein referred to, is the tissue exterior to the cambium, and wood, or xylem cylinder, that interior to it (4). The petioles of the leaves were analyzed separately in the second series (6–72 hr). Each section was analyzed for tritiated water (2) and Ca^{45} (4). Total calcium analyses were made on separate comparable plants by a chemical method (1).

The original experiment was designed to include a second part using the growth of bean plants from seed in tritiated water as an additional test for an isotope effect. The results of the first experiment, however, settled this matter against a measurable isotope effect, so that the latter experiment was unnecessary.

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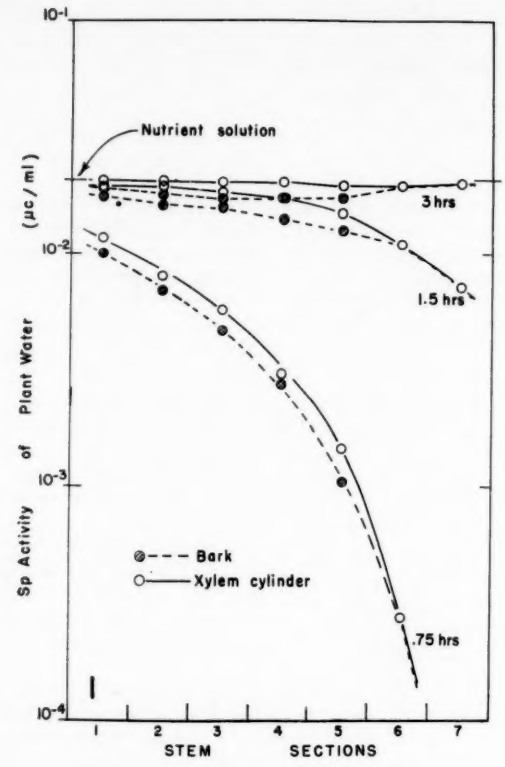
[1] Received December 23, 1960.

[2] The radioisotopes were acquired from the U.S. A.E.C., Oak Ridge, Tenn.

[3] By special assignment: Soil and Water Conservation Research Division, Western Branch, U.S.D.A. Present address, Southwest Water Conservation Laboratory, Tempe, Ariz.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1.5 hrs
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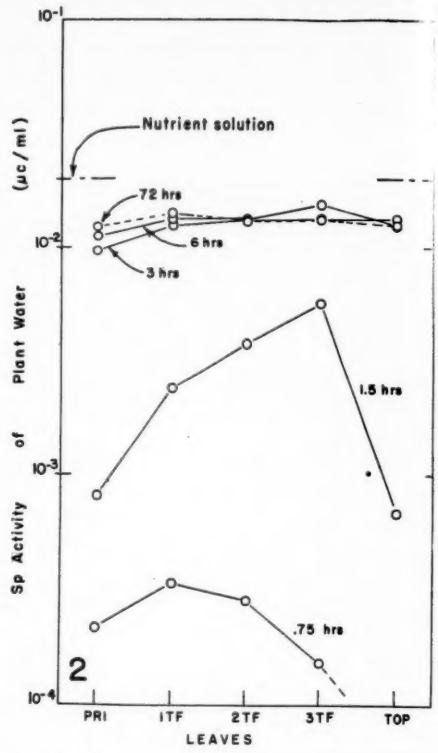
Bark
Xylem cylinder
Sp Activity of Plant Water (μc/ml)
STEM SECTIONS
1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3 hrs
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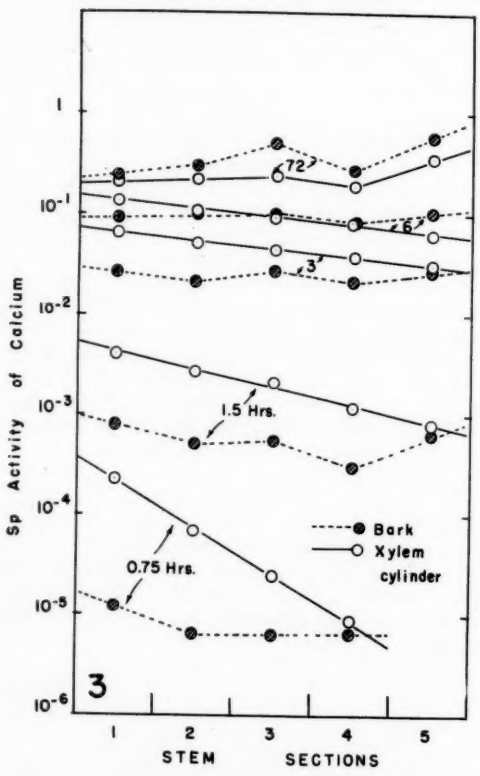
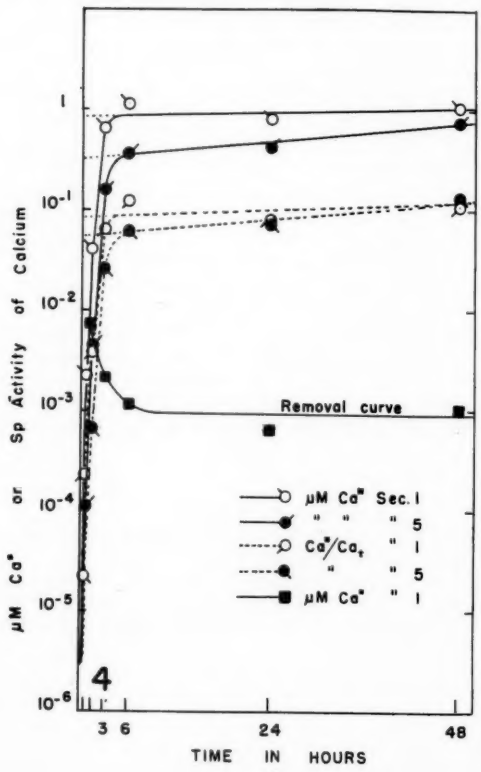
LEAVES
2


72
6
3
1.5 Hrs.
0.75 Hrs.
Bark
Xylem cylinder
Sp Activity of Calcium
STEM SECTIONS
3


Removal curve
μM Ca" Sec. 1
" " " 5
Ca"/Ca, " 1
" " 5
μM Ca" " 1
μM Ca" or Sp Activity of Calcium
TIME IN HOURS
4

The results presented herein are from single plants, each a member of a sequence of treatments. How well these results represent the progress of the two substances through the plant can be judged best by their consistency with each other rather than their conformity, as individuals, to an average, for there was no replication in that sense.

Results & Discussion

THO: Xylem Cylinder. A comparison of the specific activity of the water recovered from each plant section with the specific activity of the water in the nutrient solution was used to indicate the extent to which the labeled water from the nutrient solution had replaced the indigenous water or that initially present in the tissues. The data for individual stem sections at each time interval are shown in figure 1. These data show the measurable front of the tracer water to be near the top of the sixth section after 45 minutes. This is 8 inches from the root, and assuming, from an extrapolation, a starting point 1½ inches below this, at zero time, the measurable average rate of ascension of the tracer water in the xylem cylinder was approximately 12 inches per hour (30.5 cm/hr). The tracer water was not confined to movement in the vessels, for 50 % of the indigenous water in the central cylinder of the first stem section above the cotyledonary node was replaced by tracer water in 45 minutes. At 1½ hours (45 min later) the 50 % replacement value was 4½ inches higher up the stem. Thus, it appeared that the zone of 50 % replacement progressed up the xylem cylinder at the rate of about six inches per hour. Similarly, it can be shown that the zone of complete replacement progressed up the xylem cylinder at about three inches per hour. All water in the two lowest sections of the xylem cylinder and 98 % or more in the third and fourth sections was replaced within 3 hours. Therefore, it was concluded that while the xylem vessels may have served as the channel of least resistance for the flow of the transpiration stream, all of the xylem cylinder served to some extent for the ascent of water cf., (12).

Failure of the transpiration stream to remain confined to the xylem vessels was also shown by the distribution pattern of the tracer in the stem sections. A logarithmic plot of the specific activity of the tracer water in each stem section against the distance from the root resulted in a series of non-linear curves, figure 1. The nature of the curves and the manner in which they approached zero slope resembled the theoretical curves obtained by Horwitz (8) for the flow of a tracer through tubes in which a reversible loss through the lateral walls occurred. If the loss were irreversible, with no return movement, the curves were linear. Conformity of the present data with the theoretical curves for reversible loss corroborated the former evidence for the involvement of the entire xylem cylinder in the ascent of water.

That part of the transpiration stream moving in the vessels had little continuous molecular identity, since individual molecules exchanged out and into the stream at random. Because of the exchange, only the entity *water* could display the maximum theoretical rate of ascension in the vessels. The rate displayed by individual tracer molecules would, on the average, fall far short of this value. This can be shown as follows:

By dividing the maximum volume per hour of tracer water which had moved past the cotyledonary node by the observed rate of ascension (obtained above), the cross-sectional area of xylem cylinder necessary to carry the stream was obtained. The cross-sectional area required, for the indicated rate of ascension, was almost exactly twice the actual cross-sectional area of the xylem cylinder. This indicated the observed rate of ascension of THO, taken as an average value through 9½ inches of stem, was, at best, only half the maximum rate. The front of the THO tracer was obviously more or less continu-

Fig. 1. The specific activity of the water recovered from each section of bean stem at 0.75, 1.5, and 3 hours, after placing the plants into a nutrient medium containing THO. At 1.5 hours the specific activity in the lower part of the xylem cylinder had approached that in the nutrient medium, indicating an almost complete replacement of water. At 3 hours replacement was complete throughout. In the bark the maximum replacement was 87 %, even after 72 hours.

Fig. 2. The specific activity of the water recovered from bean leaves at 0.75, 1.5, 3, 6, and 72 hours after the plants had been placed into a nutrient medium containing THO. PRI: primary leaves, 1 TF, 2 TF, and 3 TF: first (oldest), second and third trifoliate leaves, respectively. Top: the stem and young leaves above the third trifoliate leaf. The indigenous water in the leaves was not more than 65 % replaced in 72 hours.

Fig. 3. The specific activity of the calcium from each section of bean stem at 0.75, 1.5, 3, 6, and 72 hours after placing the plants into a nutrient medium containing Ca^{45}. The specific activity in the bark exceeded that in the xylem cylinder after 6 hours, indicating a more rapid rate of replacement of indigenous calcium in this tissue.

Fig. 4. The specific activity of the calcium and the μmoles of tracer calcium in sections one and five of the xylem cylinder at various time intervals after placing the plants into the tracer solution. The uptake curves show two phases, an exchange phase and an irreversible accumulation phase. A removal curve is shown for section one of the xylem cylinder. These plants had received Ca^{45} for 1 hour only, and by subsequent exchange the tracer had fallen through almost three half values in 6 hours. The curves are characteristic of those depicting cation exchange in a variety of tissues.

ously dissipated into extra-vessel tissue as it ascended.

Further investigation into the rate of ascension of the THO tracer showed that the highest values occurred in the plants with the longest hypocotyl and basal internodes. It was in the leafy portion of the stem, and particularly in the immature portion, where the rate of ascension fell off most rapidly. The measurable ascension rate was then related to the growth form.

THO: Bark. The bark, or tissue exterior to the cambium, which contained the phloem, reached equilibration values of 86 to 93 % (avg. 88 %) in 3 hours (fig 1). At 72 hours the values were 83 to 96 % (avg. 86 %). There are two possible reasons for the failure of the bark to equilibrate: either the bark retained water which did not mix with currently incoming water, or the phloem was replenished with water which had not completely equilibrated. There are two possible sources of unequilibrated water: the first from the leaf blades (see below), and the second from the roots. Biddulph and Cory (2) have shown a downward movement of tritiated water from leaves to roots via the phloem, and Vartapetyan and Kursanov (14) have shown the roots of intact bean plants to retain unlabeled water in amounts of 47 to 59 % after having been immersed in a labeled solution (H_2O^{18}) for 24 hours. Our own results show roots of intact bean plants to be 77 % equilibrated after 24 hours' exposure in the labeled solution. A portion of the root system was then not involved in the uptake and transport of transpirational water. This portion, wherever it may have been, was not excluded as a possible source of unequilibrated water to the bark.

The exchange of all but 13 % of the water in the tissues exterior to the cambium each 3 hours showed that a part of the transpiration stream ascended in this area.

THO: Leaves. The data showing the rapidity and degree of equilibration of tracer water in the leaves and stem tip are shown in figure 2. The lower, older leaves, though they had the opportunity of acquiring tracer water ahead of the upper leaves, reached their maximum equilibration value more slowly. The maximum value, approximately 65 % of complete equilibration, was reached by all but the oldest leaves in 3 hours. These data show that the water currently lost by transpiration moved quite directly from vessels in the leaf veins to the stomata with comparatively little mixing in the mesophyll. Cline (5) and recently Vartapetyan and Kursanov (14) have both reported low equilibration rates for leaves. In Cline's report, as in ours, the leaves reached approximately 65 % of equilibration quickly and maintained this value to 72 hours—the terminal period for both experiments.

An analysis of the petioles for the 6- to 72-hour plants showed them to be from 95 to 100 % equilibrated, indicating either the passage, or very improbably, the retention of some unequilibrated water.

The retention in the leaf blades of 35 % of their indigenous water through three daily transpiration cycles suggests that in some particular part of the leaf a significant part of the water is held in a structured form. Szent-Györgyi (13) has suggested some possible roles of structured water in energy transfer systems, which would suggest an investigation of this unexchangeable water.

Ca: Xylem Cylinder. The distribution of the calcium in the various stem sections is shown in figure 3. In this figure the logarithm of the specific activity [Ca*/Ca (total)] was plotted against the distance along the stem. In contrast to the nonlinear relationship between the logarithm of the THO concentration (sp. act.) and distance along the stem, indicating reversible loss of THO from the vessels, the curves for the Ca^{45} tracer were almost linear. A linear relationship has been shown to result from an irreversible loss of tracer from the vessels (8), or from the movement of a tracer by an exchange process (2). Removal of tracer calcium from the vessels with a subsequent movement by exchange of all or a fraction of that removed would still produce a linear pattern of distribution. Movement by exchange was then definitely indicated.

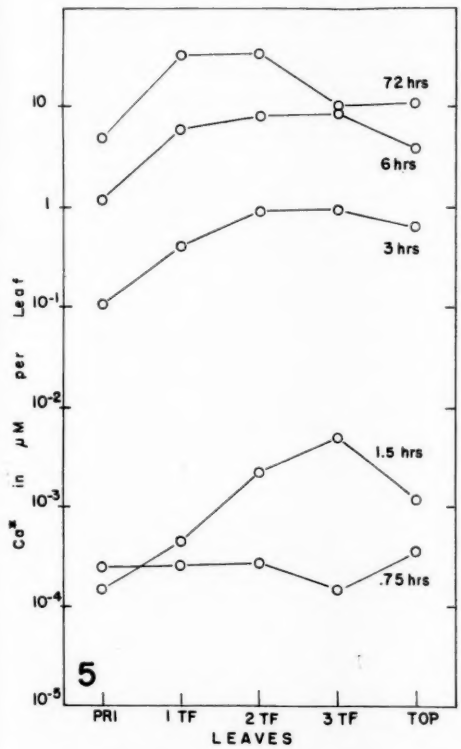
When the specific activity of the calcium in individual sections was plotted against the absorption time, the curves that resulted were identical in nature to those obtained for cation uptake in a variety of tissues (10). Specifically, the curves showed both an exchange phase and an irreversible accumulation phase (fig 4). The exchange phase reached completion in the xylem cylinder of the stem base in approximately three hours. An increase in the specific activity of the calcium in these sections showed that the exchange was accomplished by the replacement of exchangeable indigenous calcium by tracer calcium. The approximate amount of tracer calcium present on the exchange sites in sections one and five was 0.85 and 0.32 μmoles respectively, which was 8.5 and 5.5 %, respectively, of the total calcium present in those sections.

By the same process of exchange as that shown above, the exchangeable fraction of the tracer calcium could, in turn, be replaced with nontracer calcium. This was experimentally accomplished as follows: After exposure of a group of plants to tracer calcium for 1 hour, they were moved back into a nontracer solution and analyzed at intervals. The spaced intervals for analysis allowed the transpiration stream to act for varying periods and to effect the exchange in a natural way, thereby obviating the necessity of removing sections and washing out the exchangeable tracer. The removal curve of figure 4 shows the time course of the removal, by upward exchange, of the aliquot of tracer calcium, the tracer now giving way to the nontracer calcium ascending the stem. The amount of tracer fell through almost three half values (to 14 % instead of 12.5 %) in 6 hours. A further significant reduction of the 14 % could not be obtained in 72 hours, indicating that this amount

had been irreversibly accumulated and quite permanently immobilized.

Much of the indigenous calcium in the xylem cylinder remained in an unexchangeable condition, for when the exchange phase was complete, only 6 % to 9 % of that present had been replaced by tracer calcium. This increased to only 11 % to 12 % in 72 hours. This slow removal together with the retention of 14 % of the upward-moving aliquot shown above, clearly indicated the presence of a relatively permanent deposition system for a portion of the calcium which ascended the stem. The calcium oxalate crystal system, rather than metabolic accumulation in the traditional sense, appeared to be the trapping mechanism for most of this calcium. Detailed data on the Ca* oxalate system are to be presented elsewhere.

Ca: BARK. The distribution curves for Ca* in the bark sections are shown in figure 3. Initially (for the 0.75-hour period), the Ca* was low because of the time required for ascent in and removal from the xylem. At 3 hours the concentration (Ca*/g fr wt) in the bark equaled that in the adjacent xylem cylinder. At 6 hours the total amount in adjacent sections was equal and shortly thereafter the specific activities in the bark surpassed those in the xylem cylinder. Finally, at 72 hours, 50 % to 60 % of the indigenous calcium in the upper bark sections had been replaced with tracer calcium. The rapid turnover of calcium in the upper bark indicated that it, too, could serve as a channel for the ascent of calcium. This substantiates some of the contentions of Curtis (7), namely, that minerals could ascend in the bark near the stem tip.



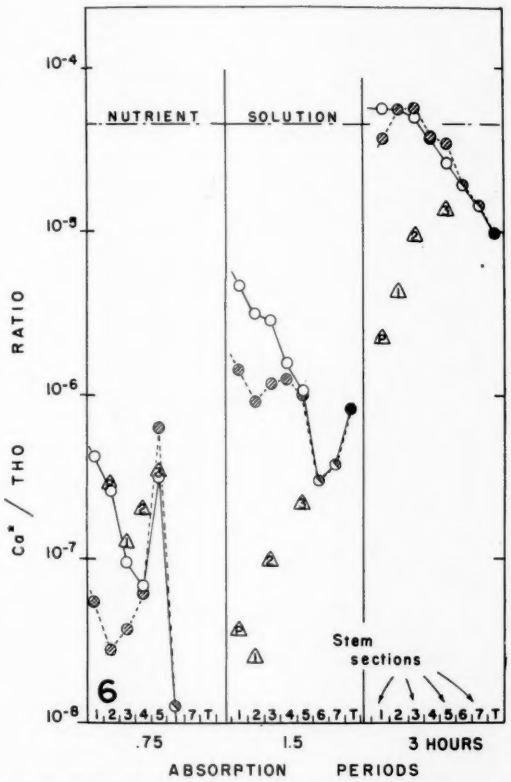


FIG. 5. The amount of tracer calcium in various bean leaves at 0.75, 1.5, 3, 6, and 72 hours after the plants had been placed into a nutrient medium containing Ca^{45}. PRI: primary leaves. 1 TF, 2 TF, 3 TF: first (lowermost), second, and third trifoliate leaves, respectively. Top: the stem and young leaves above the third trifoliate leaf.

FIG. 6. The Ca*: THO ratios for various parts of bean plants at 0.75, 1.5, and 3 hours after the plants had been placed in a nutrient medium containing Ca^{45} and THO at the ratio shown. Open circles: xylem cylinder sections. Filled circles: bark sections. Triangles with numbers: leaves; P, 1, 2, and 3 are primary and first, second, and third trifoliate leaves, respectively. The position of each leaf on the stem can be determined by relating its triangle to the stem section immediately below it.

Ca: LEAVES. The leaves did not receive tracer calcium in proportion to either size or weight during the first 6 hours; instead, position was the determining factor (fig 5). The third trifoliate leaf received the most Ca* while progressively less moved into the second, first, and the combined two primaries. This was true for periods up to and including 48 hours. As the periods lengthened, the Ca* content of the first and second trifoliate leaves approached that of the third. At 72 hours they were higher, but the 72-hour value for the third trifoliate leaf seemed low, for both the 24- and 48-hour values exceeded it. Expressed on a fresh weight basis, the ranking was not altered, but the differences merely accentuated, for the average fresh weights of the leaves were: 4.3, 3.0, 2.5, 1.9, and 1.4 g for the combined primaries, the first, second, and third trifoliate leaves and the top (0- to 6-hr averages only), respectively.

Ca*/THO RATIOS: The ratios Ca*/THO in the various leaves and stem sections for the intervals of 45 minutes, 1½ hours, and 3 hours are shown in figure 6. Similarities between the ratios for the leaves and the sections of xylem cylinder from which they diverged were found for only the 45-minute period. This indicated that the first influx of tracers into both stems and leaves was largely confined to a movement in the xylem vessels. Subsequent movement into leaves must continue via the xylem vessels as no extensive extra-vascular tissue extends through the petiole.

At 1½ hours the Ca*/THO ratio for the first (lowermost) section of xylem cylinder was higher by a factor of ten than for the fifth section. This was because the THO was approaching its equilibrium value in all sections, while the Ca* was just entering into the stem base in significant quantity.

The Ca*/THO ratios for the leaves were much lower than those for the stem sections. Among leaves the lowest ratio, that for the primary leaf, was smaller by a factor of ten than the highest ratio, that for the third trifoliate leaf. In order for a more dilute transpiration stream to enter the lower leaves than moved into the upper ones, the removal of some Ca* from the leaf traces leading to the lower leaves would be required. The leaf traces in the bean plant traverse a full node after diverging from the vascular cylinder. It was during the passage through this internode that the Ca* content of the transpiration stream was reduced. A significant and possibly a major portion of that which left the xylem was found in the adjacent phloem. The transfer occurred predominantly at the node of divergence of the leaf, and some Ca* extended downward in the phloem traces as though it had moved in the assimilate stream. This mineral extracting mechanism near the pulvinus of mature leaves augments exchange toward the apex by returning to the stem a portion of the minerals carried in the leaf traces. Figure 7 shows Ca^{45} and Sr^{89} radioautograms of the bark wherein the lateral transfer at the nodes is evident. A similar transfer has been reported earlier for P^{32} and S^{35} (3). The details of the transfer mechanism will be reported at a later time.

At the 3-hour period the Ca*/THO ratios for the stem sections had approached their original ratio in the nutrient medium. This was because the THO values had been at equilibrium for approximately 1½ hours while the Ca* was just completing its exchange up the stem. Following this period, increases in the ratio were slower and were due to augmentation of Ca* to that fraction which was irreversibly accumulated.

It is now quite clear that Ca*/THO ratios should not be interpreted as representing solution concentrations. Neither should the value, mineral absorbed/water transpired, be used to convey the impression that the two move only *en masse* and as a solution corresponding to this particular concentration, cf., Hylmo (9), and Russell and Shorrocks (11). Our data show the xylem cylinder of the plant stem fundamentally to operate as an exchange column through which narrow vessels with porous walls permit solvent movement at somewhat less resistance than is encountered in the ground tissue as a whole.

A translocation mechanism involving an exchange process explains the observed unequal rates of ascent of Ca* and THO, and their different distribution characteristics in different plant parts, more successfully than a mechanism based on mass flow. With mass flow there is no flexibility or opportunity for control over delivery save that inherent in transpirational loss from individual tissues or organs. On the other hand, ascent by exchange would be dependent upon both the nature of the ion and of the exchange sites, the current loading of the sites, and the rapidity of removal from the exchange system. Removal at the stem apex, and other metabolically active places, would be into new space created by either growth or utilization thus bringing control of ascent more nearly under the influence of tissue metabolism at each receiving site than could be so with a mass flow controlled only by transpiration. Such an exchange system, composed of a variety of biocolloids over which movement is actuated by water of the transpiration stream, offers the flexibility and the control mechanisms necessary for explaining most of the observed characteristics of mineral ascent.

SUMMARY

The degree of dependence between water movement and calcium movement was determined by the sequence and pattern of entrance of THO and Ca^{45}, used simultaneously, into various tissues and organs of the bean plant. The root-water reached only 77 % of equilibration with nutrient water in 24 hours while equilibration in the xylem cylinder of the stem was complete to the third trifoliate leaf in 3 hours. The bark and leaves reached 87 % and 65 %, respectively, of equilibration in 3 hours and neither exceeded this value in 72 hours.

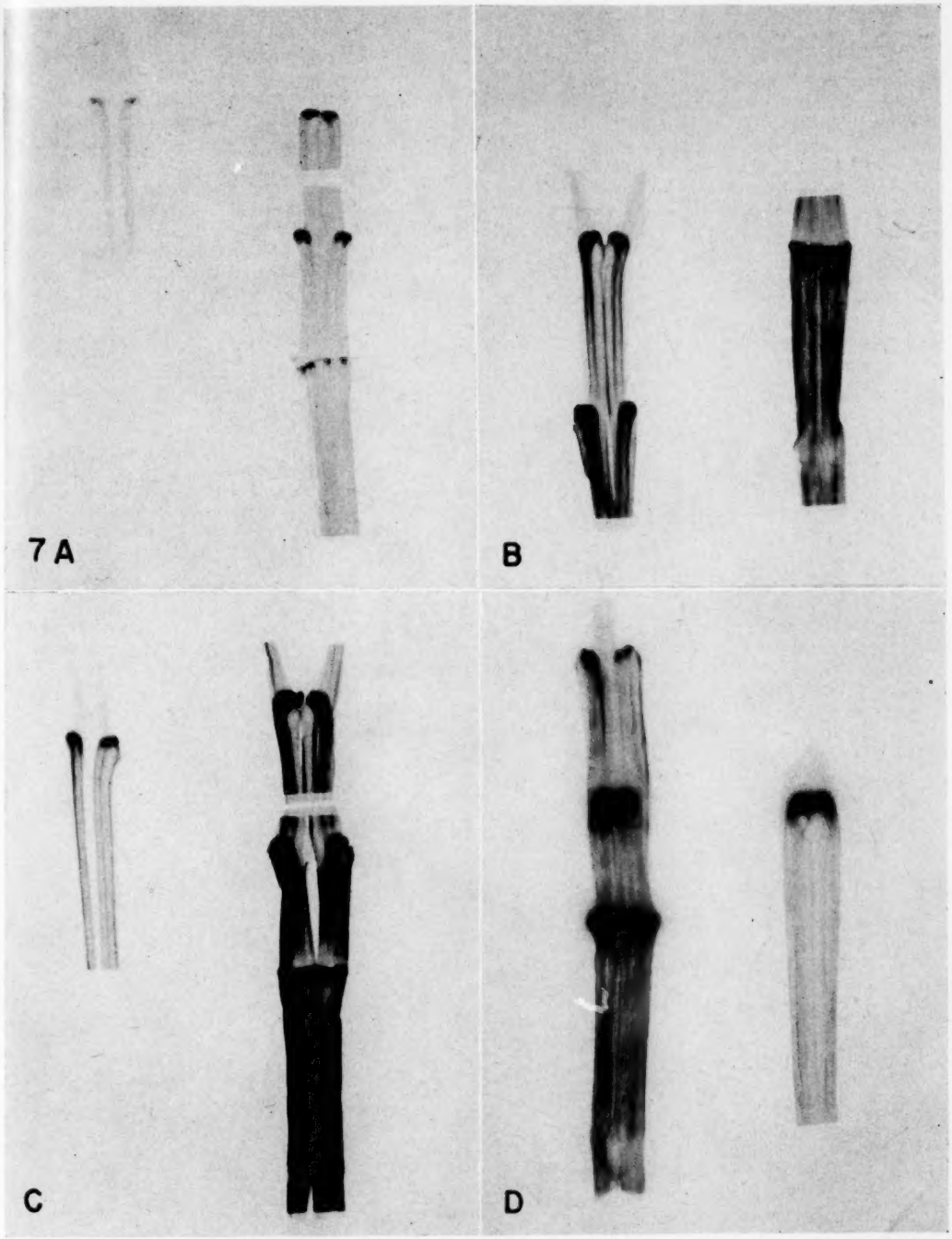


FIG. 7. Ca^{45} radioautograms of frozen-dried bark of bean stems. The plants remained in a half-strength Hoagland solution containing Ca^{45} for A, 0.75 hours; B, 1.5 hours; and C, 3 hours, respectively, prior to stripping the bark from the xylem cylinder. The tissues were frozen in isopentane chilled in liquid N_2. The base of the section of bark on the left joins the top of the section on the right to show the primary, first, second, and third (except B) trifoliate leaf nodes, respectively. A distinct transfer of Ca^{45} from the xylem component of the leaf traces to the phloem is evident at each node. D is a similar radioautogram of Sr^{89} corresponding to B, except the position of bark sections is reversed.

The time course curve for Ca^{45} entry into specific stem sections showed that entrance consisted of two phases: a reversible exchange phase and an irreversible accumulation phase. The exchange phase was complete in 3 hours and the exchangeable fraction constituted less than 10 % of the total calcium. The Ca^{45} on exchange sites could, in turn, he exchanged up the stem, the loss curve for the tracer falling through three half values in 6 hours. The non-exchangeable Ca^{45} was mostly in the calcium oxalate crystal system, the low solubility constituting the driving force for accumulation.

The ratio of Ca*/THO in leaves increased stepwise from the primary leaves (lowest values) to the third trifoliate leaves (highest values). Radioautograms of bark tissues showed a transfer of Ca* from the xylem of leaf traces to the adjacent phloem sufficient to account for the low Ca*/THO ratios in older leaves.

The data showed the xylem cylinder of the bean stem fundamentally to operate as an exchange column for calcium. *En masse* flow in the vessels was inadequate for explaining the rapid deep-seated exchanges observed for this tracer.

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THE ESSENTIAL ROLE OF CALCIUM IN SELECTIVE CATION TRANSPORT BY PLANT CELLS [1]

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The mechanisms whereby plant cells absorb inorganic ions from the external media are selective: they discriminate among different ions. Particularly intriguing is the discrimination between potassium and sodium ions. Most plant cells accumulate much higher concentrations of potassium than of sodium ions, even from media in which the sodium concentration greatly exceeds that of potassium.

Perhaps the best way to investigate these specificities is to study the manner in which different ions affect each other's absorption. In a previous paper (4) experiments were described demonstrating that in excised barley roots potassium and rubidium compete with each other in an identical absorption mechanism. At moderate concentrations, neither sodium nor lithium ions competed for the potassium-rubidium transport mechanism. The deduction was that there are specific ion binding sites on carrier molecules which effect the transport of the ions across cellular membranes. Potassium and rubidium ions are bound and transported by sites which fail to discriminate between them, hence these ions compete with each other for identical carrier sites. Sodium and lithium are not bound by the potassium-rubidium sites, hence do not compete with potassium and rubidium but are transported by separate sites.

In this paper experiments with barley roots are presented which show that calcium ions are essential for the integrity of the selective absorption mechanism referred to, and information is given about some quantitative features of the calcium effect.

MATERIALS & METHODS

The distilled water piped into the laboratory was passed through a mixed ion exchange column [2] to free it from heavy metal and other impurities. This purified water was stored in pyrex carboys. Glassware was rinsed with about 0.5 M HCl, followed by exhaustive rinsing with distilled water. Salts used were C.P. grade. Radioactive Rb^{86} and K^{42} were obtained from Oak Ridge National Laboratory, and Na^{22} from Nuclear Science & Engineering Corp., Pittsburgh 36, Pa. The stock solutions were labeled to the extent of about 0.002 μc per μmole.

PREPARATION OF ROOT MATERIAL. Seeds of barley, *Hordeum vulgare* L. var. Arivat, were allowed to germinate for 24 hours in 1 liter aerated water in the dark, at room temperature (about 20° C). The water was renewed once during this time, 19 hours after the start of the germination period. The germinating seeds were spread on a layer of cheesecloth which had been washed and rinsed with distilled water. The cheesecloth was supported by a stainless steel screen about one centimeter above the surface of 4 liters of 2×10^{-4} M $CaSO_4$ solution in a 4-liter pyrex beaker. A second cheesecloth was spread over the seeds. The corners of both cheesecloths dipped into the solution. An inverted watchglass, 17.5 cm in diameter, was placed on the beaker. The top cheesecloth was removed as the germinating seedlings pushed it up.

This assembly was placed in the dark, at room temperature. The solution was aerated continuously. Three days after the seeds were planted on the cheesecloth, the stainless steel screen with the seedlings was removed from the beaker, the roots were rinsed in two changes of water, and the assembly put on a beaker with fresh solution. The watchglass was not replaced. Two days after this, the roots were rinsed repeatedly in water, excised just below the screen, rinsed several more times, and suspended in about four liters of water.

ABSORPTION EXPERIMENTS. For the experiments proper, roots were gently blotted on dry cheesecloth which had been washed and rinsed with distilled water. One-gram samples were weighed out on a torsion balance and transferred to 100 ml water in 30×300 mm culture tubes. The tubes were placed in a waterbath maintained at 30° C, and the water in the tubes was aerated. To start the absorption period proper, each tube in turn was removed from the bath, the water was decanted, the roots were rinsed twice with about 100 ml water each time, and 100 ml of prepared experimental solution were then poured in. The tubes were replaced in the bath, and the solution was kept aerated.

[1] Received January 9, 1961.

[2] Bantam Barnstead Demineralizer, made by Barnstead Still and Sterilizer Co., Boston 31, Mass.

The absorption period was discontinued by decanting the experimental solution and rinsing the roots for 1 minute with a total of at least 500 ml water.

All salts of the experimental solutions were chlorides. The initial pH (unbuffered) was 4.9, and this changed by no more than 0.2 pH units during the experimental runs. The roots contained initially no detectable Rb, less than 1 μmole Na per gram fresh weight, and about 15 μmole K. These two values varied slightly from experiment to experiment, although all seed used came from the same batch.

RADIOACTIVE ASSAY. The root samples were transferred to 1-inch × 5/16-inch nickel-plated planchets, dried, and ashed at 500° C. A little water and one drop detergent solution were added to the ash and the samples dried under infra-red lamps. The samples were counted with a thin-window (1.4 mg/cm²) Geiger-Mueller tube connected to a conventional scaler. Each sample was counted twice, to at least 3,000 counts each time. In most experiments, samples were counted to 10,000 counts each time. Samples of an appropriate dilution of the radioactive stock solution were pipetted into counting cups and after addition of a drop of detergent were dried under infra-red lamps.

FLAME PHOTOMETRIC ASSAY. The root samples were transferred to 30-ml pyrex beakers, dried, and ashed at 500° C. The ash was taken up in 0.16 N HNO_3, and assayed in a Beckman Model DU spectrophotometer with photomultiplier and flame attachment. Standard curves were prepared from known solutions of the salts in 0.16 N HNO_3. Potassium was assayed at 766 mμ, sodium at 589 mμ, and rubidium at 780 mμ.

TABLE I

EFFECTIVENESS OF DIVALENT CATIONS IN REVERSING INHIBITION OF Rb ABSORPTION BY Na IONS*

Na Conc, mM	Divalent cation	Rb Absorption		Rank of divalent cation
		μmole/g/hr	% of control**	
0	...	6.6	...	...
0	Ca	6.9	...	...
20	...	2.0	100	...
20	Mg	2.3	115	8
20	Ca	4.3	215	1
20	Sr	3.2	160	5
20	Ba	3.5	175	4
20	Mn	4.0	200	2
20	Co	2.8	140	6
20	Ni	2.6	130	7
20	Zn	3.7	185	3

* Concentration of Rb: 1 mM. Concentration of divalent cations: 0.05 mM; time: 60 minutes.

** The sample with Na present at 20 mM concentration, but without added divalent cation, is considered the control (100 %)

CONSISTENCY & RELIABILITY OF RESULTS. For each experiment reported, one or several others were done with or without modifications. No results were obtained that were inconsistent with those reported.

RESULTS

The effect of Na on absorption of Rb during a 60-minute period is shown in figure 1[3]. In the absence of Ca, Rb absorption from a 1 mM solution of RbCl was progressively diminished by increasing concentrations of NaCl. In the presence of Ca, the effect of Na was quite different, and this typical response to Na was shown in experiments both on Rb and K absorption (figs 1, 2, 3, 5). There always is a slight inhibition of absorption at 1 to 2 mM Na. Between 5 and 10 mM Na, absorption of K or Rb rises to almost the control (no Na) value, and at still higher Na concentrations declines slightly.

This marked indifference of K and Rb absorption to even high concentrations of Na, in the presence of Ca, is in conspicuous contrast to the pronounced inhibition of K absorption by Rb (fig 2), and of Rb absorption by K (fig 3). Both of these experiments lasted 60 minutes, and the K and Rb concentration was 1 mM. Calcium was present at a concentration of 0.05 mM.

In the previous experiments, the concentration of Ca was 0.05 mM. The effect of varying the Ca concentration over a wide range is shown in figure 4. The Rb concentration was 1 mM and that of Na 20 mM throughout. Calcium concentrations are plotted on a logarithmic scale, experimental points lying at full powers of ten and three times these values. At the Na concentration used (20 mM), Rb absorption from a 1 mM solution was reduced to 2.1 μmole/g/hour, in the absence of Ca, and there was no effect from Ca at 1×10^{-6} N. At higher Ca concentrations Ca became increasingly effective in reversing the inhibition due to Na, Rb absorption rising most steeply at about 1×10^{-4} N Ca, and leveling off at 3×10^{-4} to 1×10^{-3} N Ca. In a similar experiment with K as the ion absorbed, the Na concentration was 10 mM, and the absorption of K from a 5 mM solution was determined as a function of the Ca concentration over the same range. Assay in this experiment was by flame photometry. The response in terms of K absorption paralleled that in the present experiment on absorption of Rb.

Is calcium the only divalent cation capable of protecting the K-Rb absorption mechanism against the Na inhibition? Table I gives the results of an experiment in which the effectiveness of eight divalent cations was tested. The first two lines show results obtained without Na in the solution. In the absence

[3] Asterisks in the figures denote radioactively labeled ions.

of Na, Ca at 0.05 mM does not appreciably increase the amount of Rb absorbed. The severe inhibition of Rb absorption by Na (line 3) is reversed by Ca (line 5) and the other divalent cations tested, to varying degrees. Calcium is the most effective ion. The last column of table I ranks all ions tested in order of their effectiveness.

A curious result (table I) was the ineffectiveness of the Mg ion in affording protection against the Na inhibition of Rb absorption. The value to which the Na inhibition reduced Rb absorption, viz. 2.0 μmole, was more than doubled in the presence of Ca, but Mg at the same concentration increased it by merely 15 %. No other ion, among those tested, was equally ineffective. Figure 5 shows the results of an experiment in which both Ca and Mg were studied in their relative effectiveness in counteracting the Na inhibition of K absorption. The roots absorbed from 1 mM solutions of K, for 60 minutes. At the lowest Na concentrations Mg had some effect, but at the higher concentrations of Na the effectiveness of Mg declined and at 20 mM Na, K absorption in the presence of Mg was not significantly higher than in absence of any divalent cation. Calcium largely protected against Na inhibition, as usual.

The effect of time was studied in an experiment in which roots absorbed Rb from a 1 mM solution in the presence, throughout, of 20 mM Na. In one set, Ca was present at 0.1 mM from the beginning. Another set had no calcium. A third was without Ca for the first 24 minutes of the absorption period, then Ca was added in 0.1 mM concentration. The results are shown in figure 6.

The foremost features in this experiment are as

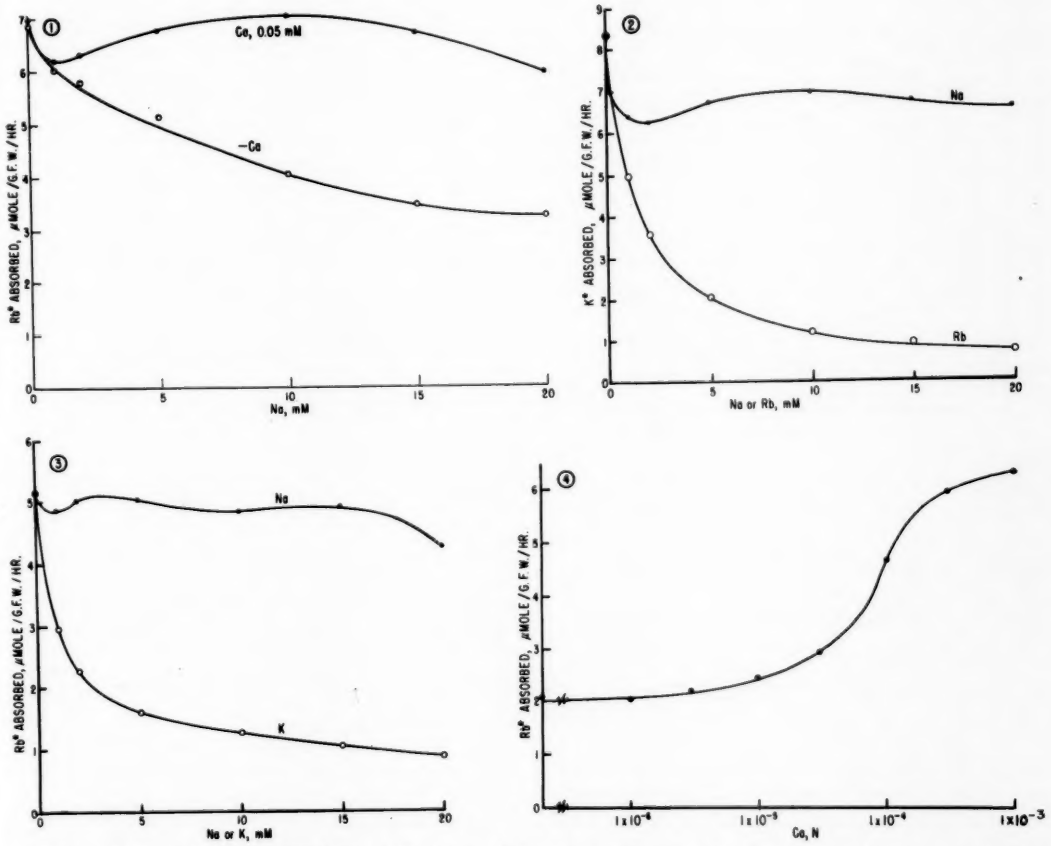


FIG. 1. Effect of Na on absorption of Rb in the absence and presence of Ca. Rb: 1 mM; time: 60 minutes.

FIG. 2. Effect of Na and of Rb on absorption of K in the presence of 0.05 mM Ca throughout. K: 1 mM; time: 60 minutes.

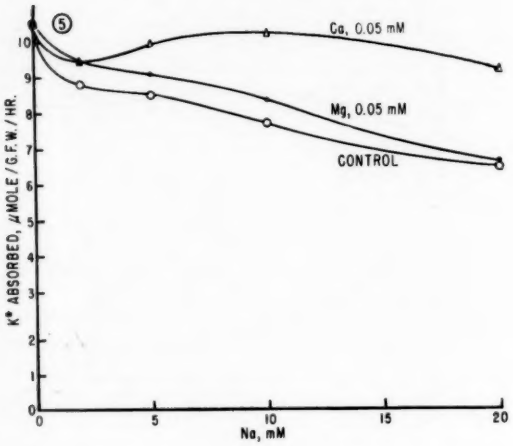
FIG. 3. Effect of Na and of K on absorption of Rb in the presence of 0.05 mM Ca throughout. Rb: 1 mM; time: 60 minutes.

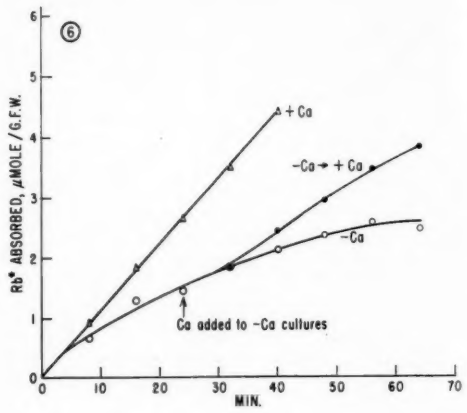
FIG. 4. Effect of Ca concentration in reversing the inhibition of Rb absorption by Na. Rb: 1 mM; Na: 20 mM; time: 60 minutes.

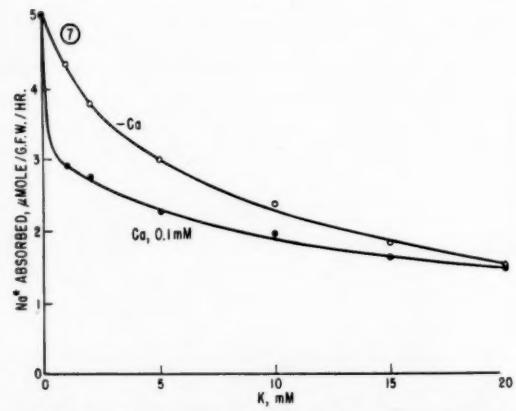
follows: In the presence of Ca, Rb absorption was a strictly linear function of time, extrapolating to zero at zero time. Without Ca, the rate of absorption declined all along. Upon addition of Ca to the solutions lacking Ca there was no immediate effect on the rate of Rb absorption, but after about an 8-minute lag the rate of Rb absorption increased. The recovery was not complete, however, in that these roots did not absorb Rb as fast as did those that had Ca present from the beginning of the absorption period, nor was the rate of absorption constant.

In the previous experiments it has been shown that in the presence of Ca at concentrations of 0.05 to 0.1 mM, Na over a wide range of concentrations fails to compete with either K or Rb in the absorption process (figs 1, 2, 3, 5), while K and Rb do mutually compete (figs 2, 3). What is the converse effect, that of K or Rb upon the absorption of Na? Figure 7 shows the results of an experiment in which Na absorption from a 1 mM solution was studied as affected by increasing concentrations of K, in the absence and presence of Ca at 0.1 mM concentration. The absorption period was 60 minutes.

In the absence of Ca, Na absorption progressively declines with increasing K concentration, much as Rb absorption and K absorption are decreased by progressively higher concentrations of Na, without added Ca (figs 1 & 5). In the presence of Ca, on the other hand, there is a sharp drop in Na absorption at 1 mM K. At higher K concentrations, Na uptake decreases much more gradually, and virtually levels off at the highest K concentrations tested. These features are brought out even more clearly in the experiment shown in figure 8, identical with the previous one in every respect except that the Na concentration was 5 mM.







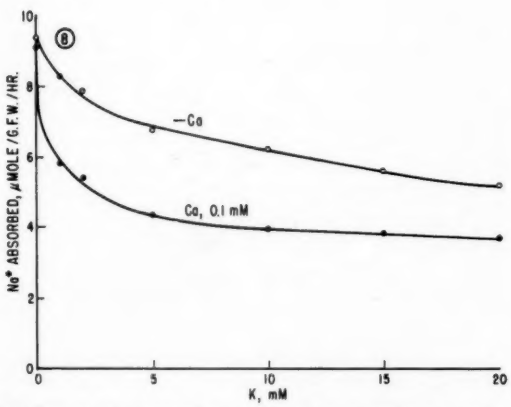


FIG. 5. Effectiveness of Ca and Mg in reversing the inhibition of K absorption by Na. K: 1 mM; time: 60 minutes.

FIG. 6. Time course of Rb absorption in the presence of Na, and the effect of Ca. Rb: 1 mM; Na: 20 mM; Ca where present: 0.1 mM.

FIG. 7. Effect of K on absorption of Na in the absence and presence of Ca. Na: 1 mM; time: 60 minutes.

FIG. 8. Effect of K on absorption of Na in the absence and presence of Ca. Na: 5 mM; time: 60 minutes.

TABLE II

ABSORPTION OF Rb & Na AS A FUNCTION OF Na CONCENTRATION IN ABSENCE & PRESENCE OF Ca*

Na Conc mM	Rb Absorbed, μmole		Na Absorbed, μmole	
	−Ca	+Ca	−Ca	+Ca
0	6.5	6.6	...	...
5	5.0	6.4	5.4	3.9
10	4.3	6.6	8.7	5.7
20	3.5	6.8	13.5	8.0

* Concentration of Rb: 1 mM throughout. Calcium, where present: 0.1 mM. Absorption period: 60 minutes. Experiment done in duplicate, one set with Rb* for radioactive assay, the other with Rb for Na assay.

In table II the results of an experiment are presented in which the absorption of Rb and Na was measured both in the absence and presence of Ca at 0.1 mM. The Rb concentration was 1 mM; Na ranged from zero to 20 mM. In stabilizing the K-Rb absorption mechanism against inhibition by Na, Ca appreciably reduces the absorption of Na (cf. figs 7 & 8).

DISCUSSION

The results confirm and extend previous conclusions (4): K and Rb ions are mutually competitive, Na does not compete in at least one K-Rb absorption mechanism. In the present work Ca is shown to be essential for the maintenance of the integrity of this selective ion transport mechanism.

In particular, Ca prevents Na from competing with K and Rb at the higher Na concentrations. In the earlier experiments, Na at low concentration (up to 10 mM) did not compete with Rb, but it did compete at higher concentrations. It is shown here that without Ca, the selectivity between K and Rb on the one hand and Na on the other breaks down even at low concentrations of Na. Evidently enough endogenous Ca was present in the earlier experiments to permit demonstration of the relative indifference of the K-Rb transport mechanism to Na. In the present experiments another variety of barley was used, the volumes of experimental solution were larger, and the roots were much more exhaustively rinsed before the absorption period. These factors made for a more thorough removal of diffusible Ca from the tissue, and permitted demonstration of the essentiality of Ca in the selective ion transport mechanism.

In analyzing the results, a convention adopted earlier (4) will be used. The ion whose absorption is discussed at the moment will be called the substrate ion, and the ion whose effect on the absorption of the substrate ion is being tested will be the interfering ion. As a matter of fact, both the substrate and the interfering ions are absorbed, and the terminology is used for convenience only.

The results shown (figs 2 & 3) are consistent with the previous interpretation (4) that K and Rb are transported by identical carrier sites and compete with each other for these sites. With K as the substrate ion, Rb at increasing concentrations progressively diminishes the absorption of K indicating the progressive displacement of K ions by Rb ions from the available carrier sites (fig 2). Competition for identical sites is mutual, by definition, and figure 3 shows that K in quite similar manner reduces the absorption of Rb. The identity of the K and Rb transport mechanisms is further brought out by the fact that the absorption of both is affected in similar manner by Na ions, in the presence of Ca (figs 1, 2, 3, 5). One cannot tell, without reading the legends, whether a given graph depicts the effect of Na on K or Rb absorption.

The K-Rb carrier mechanism is highly indifferent to even a 20-fold excess of sodium in the solution, but only in the presence of Ca (figs 1, 5). Evidently under these conditions Na, over a wide range of concentrations, fails to compete for the K-Rb carrier sites. This difference between Na on the one hand, and K-Rb on the other, is strictly a phenomenon of cation selectivity, distinct from any anion effect. The concentration of Cl ions accompanying the interfering cation increased progressively in precisely the same manner, whether Rb or Na was the interfering cation (fig 2). The sharp contrast between the effects of these two chlorides on K absorption was therefore due entirely to the respective cations. The same considerations hold for the findings shown in figure 3.

Calcium has been shown by Viets (14) and others to accelerate the absorption of various ions by plant tissues, including the absorption of K (8, 11, 14) and Rb (3) by barley roots, the absorption of Rb by mung bean roots (13) and the absorption of K by corn and soybean roots (10). Is the Ca effect described in the present paper identical with this earlier effect of Ca and other divalent and trivalent cations on ion absorption? The answer to this question is no, for several reasons.

I. The effect described heretofore of accelerated ion absorption is produced not only by Ca ions but by several other divalent cations as well, including Mg ions. In all but one of Viets's (14) experiments dealing with the comparison of various divalent cations, Mg was second only to Ca in accelerating ion absorption by barley roots. Tanada (13) also found that results obtained with Mg were essentially similar to those obtained with Ca in experiments on the influence of these ions on the absorption of Rb and H_2PO_4 by mung bean roots. In the present experiments, Mg was the least effective among the divalent ions tested (table I, fig 5).

II. The Ca effect described in the present paper approaches a maximum at Ca concentrations on the order of 3×10^{-4} to 1×10^{-3} N, and is about half maximal at 1×10^{-4} N Ca (fig 4). The acceleration

of ion absorption described by Viets (14) and others (3, 11, 13) was caused by Ca at much higher concentrations, saturating Ca concentrations being usually on the order of 1×10^{-2} N or even higher.

III. The previous reports referred to experiments in which the experimental solutions contained two salts: the salt of the substrate ion whose absorption was studied, and a salt of Ca or some other divalent or trivalent cation. Under similar conditions, i.e., in the absence of Na, Ca at concentrations of 0.05 mM or 0.1 mM did not accelerate the absorption of Rb significantly (table I, 1st 2 lines; table II, 1st line; figs 1, 5). Only when the absorption of K or Rb was inhibited by Na did Ca at these concentrations increase their absorption.

It is concluded that the Ca effect described here differs from that discussed by Viets and others. Their experiments showed that divalent cations caused an acceleration of ion absorption per se. The present experiments describe a reversal, by Ca and certain other divalent cations, of the inhibition of K-Rb transport by Na. For this reversal, Ca is effective at much lower concentrations than those required for straightforward acceleration of ion absorption, and Mg is largely ineffective.

In the presence of Na at 20 mM, the rate of Rb absorption steadily declined if no Ca was present, but remained constant in the presence of 0.1 mM Ca (fig 6). The findings suggest a progressive derangement of the K-Rb transport mechanism by Na, in the absence of Ca. Recovery from this Na- induced impairment upon addition of Ca was neither immediate nor complete. It took more than 8 minutes after addition of the Ca before the rate of Rb absorption increased, and even then it remained lower and less steady than the rate of absorption by those roots that had Ca present from the beginning. The lag in the recovery after addition of Ca was slightly shorter in another experiment similar to that shown in figure 6, being a little less than 7 minutes. Such a lag is not surprising in view of the fact that the initial penetration of divalent cations into the tissue is fairly slow, about 75 % equilibration being reached in 8 minutes (5).

Inasmuch as Na, in the presence of Ca, failed to compete with K-Rb over a wide range of concentrations, experiments were done to examine the reciprocal effect, viz., the influence of K-Rb on the absorption of Na. Absorption of Na was depressed by K more severely in the presence of Ca than in its absence, but comparison is invited of this K effect on Na absorption (figs 7 & 8) with the straightforward competitive situation, as when K competes with Rb (fig 3), or Rb with K (fig 2). The latter responses are consistent with the previous interpretation (4) that these two ions, K and Rb, displace each other from identical carrier sites. As the ratio of interfering to substrate ions is progressively raised, complete displacement of the substrate ions by the interfering ones is asymptotically approached.

Clearly, such a simple competitive situation does not prevail when K interferes with the absorption of Na, in the presence of Ca (figs 7 & 8). At low K concentrations there is a pronounced inhibition of Na absorption, but at the higher concentrations of K the response of Na absorption to added increments of K becomes very slight, especially when the Na concentration is 5 mM (fig 8). This is interpreted as indicating the existence of at least two Na carrier sites. The operation of one of these is inhibited by K, the other one is quite indifferent to K and accounts for the absorption of Na at the higher concentrations of K.

A double mechanism is suggested for K-Rb absorption as well. Figure 2 shows that at low concentrations, Na interfered somewhat with K absorption, indicating a Na-sensitive K transport mechanism, but the mechanism effecting K absorption at the higher Na concentrations was quite indifferent to Na. The same phenomenon is apparent to a lesser extent in the other experiments on Na interference with K-Rb absorption, in the presence of Ca. It is more pronounced when the K-Rb concentration is higher (see 4, fig 1).

In these experiments it has been shown that from solutions containing both K (or Rb) and Na, the tissue absorbs relatively more K (or Rb) and relatively less Na in the presence of Ca than in its absence. It might be tempting to advance the explanation that Ca minimizes Na interference with K-Rb transport by excluding Na, thus preempting carrier sites for K-Rb transport by preventing Na from having access to them. However, the facts do not support the idea of a simple one-to-one-relationship. Table II shows, as did the experiments presented in figures 1, 2, 3, and 5, that Ca reversed the progressive inhibition of the K-Rb transport by ever larger concentrations of Na. Nevertheless, absorption of Na, though less than in the absence of Ca, increased with increasing Na concentrations even in the presence of Ca, and at the highest Na concentration, 20 mM, exceeded the absorption of Rb. At this concentration Na, in the absence of Ca, caused a drop in Rb uptake of 3 μmoles, from 6.5 to 3.5 μmoles, while 13.5 μmoles Na were absorbed. In the presence of Ca, Rb absorption was essentially unaffected, and 8.0 μmoles Na were absorbed at the highest Na concentration.

The following hypothesis is consistent with the facts. Two species of carrier sites transport K-Rb. One of these, A, with a relatively low affinity for K-Rb ions, is inhibited by Na (fig 2), and more severely so, the higher the K-Rb concentration (4, fig 1). This latter feature is due to the fact that this low-affinity site accounts for a greater proportion of the total K-Rb transport at the higher K-Rb concentrations. The inhibition by Na of K-Rb transport by this site is mitigated but not completely reversed by Ca (figs 1, 5). The other site, B, has a higher affinity for K and, in the presence but not in the ab-

sence of Ca, is largely unaffected by Na. This is the site responsible for the lack of inhibition of K-Rb transport at the higher Na concentrations when Ca is present (figs 1, 2, 3, 5).

Two carrier sites are indicated for the transport of Na as well. In the absence of Ca, Na is transported by a site, C, with relatively low affinity for the ion. When Ca is present this site is largely preempted by K (figs 7, 8, over the lower range of K concentrations). Another part of the Na transport is through a site, D, with high affinity for Na ions and largely indifferent to K, in the presence of Ca (figs 7, 8, at the higher concentrations of K).

A careful consideration of all the data suggests that the K-Rb transporting site A may be identical with the Na transporting site C, and to that extent there exists indeed a mechanism common to K-Rb and Na transport. This site has a much higher affinity for K than for Na when Ca is present, hence the sharp drop in Na absorption at even low K concentrations, in the presence of Ca (figs 7, 8).

The significance of these findings lies in the fact that plant cells under normal physiological conditions absorb K and Na *in the presence of Ca*. Only the presence of sufficient Ca of endogenous origin permitted the earlier demonstration (4) of distinct transport sites for K-Rb and Na, respectively. When the precautions described here are taken, this selectivity breaks down if Ca is omitted from the solutions.

This conclusion probably applies to plant cells generally. The red alga, *Porphyra perforata*, differs in its K and Na transport from barley roots in that it accumulates K and extrudes Na, but here, too, Ca is essential in maintaining the integrity of the distinct K and Na transport mechanisms (2). Jacobson et al (9) have shown that the roots of plants of several species absorb more K and less Na in the presence of Ca than in its absence. A general impairment of ion transport through removal of Ca has been described by Hanson (6) and Helder (7), but their studies did not deal with the selective feature of K-Rb vs. Na transport described here.

Certain other divalent cations besides Ca, notably Mn, can partially reverse the inhibition of K-Rb absorption by Na (table I), yet Ca probably is the ion chiefly responsible for the maintenance of ion selectivity under normal, physiological conditions. Physiological concentrations of such divalent micronutrient cations as Mn and Zn are on the order of micromoles per liter, but in this range of concentrations even Ca was without much effect (fig 4). Manganese and zinc, when tested at a concentration of 5 μM (1×10^{-5} N), were about as effective as Ca at that concentration (cf. fig 4). Only Mg, among the divalent cations tested, is present in natural substrates at concentrations of the same order of magnitude as Ca ions, and it was quite ineffective in reversing the inhibition, by Na, of K-Rb absorption (table I, fig 5).

The present findings strengthen the earlier conclusion (4) that in barley roots and probably quite generally in plant cells, there are carrier sites involved in the transport of K-Rb which are distinct from others that transport Na ions. The opposite view, that there is a single common mechanism responsible for the absorption of these cations, has been discussed in the literature (1, 9, 12). Particularly relevant are the conclusions of Conway and Duggan (1) on ion absorption by yeast because they used the same kinetic treatment (4) that had led to the finding of separate mechanisms of K and Na absorption in barley roots. They concluded that in yeast, absorption of K and Na is by one and the same carrier mechanism. These experiments with yeast were done at high concentrations of the monovalent cations (up to 100 mM Na), in the absence of divalent cations. It is thus quite possible that different results would have been obtained under more nearly physiological conditions, which include the presence of Ca or other divalent cations.

The present results cannot be explained by a scheme of simple mutual competition by K-Rb and Na for a single common carrier site. The operation of at least three sites is demonstrated of which one is highly selective for K-Rb and another for Na. The present findings confirm the validity of the "enzyme kinetic" analysis of absorption rates which initially led to the conclusion of separate K-Rb and Na transport sites (4).

Summary

Excised barley roots, rinsed thoroughly free of diffusible Ca, absorbed ions from solutions containing KCl or RbCl or NaCl, or combinations of these, in the presence or absence of Ca or other divalent cations, for periods of 60 minutes in most experiments.

In the absence of Ca, Na interferes with K absorption, and K with Na absorption, at all concentration ratios examined. In the presence of Ca, Na interferes slightly with K absorption at low Na concentrations (1–2 mM), but at progressively higher concentrations of Na there is little or no further inhibition of K absorption.

In the presence of Ca, K inhibits absorption of Na at low K concentrations, but as the K concentrations are raised inhibition of Na absorption per added increment of K becomes very slight.

Potassium and rubidium are mutually competitive in the presence of Ca.

Calcium is maximally effective in maintaining the integrity of the selective absorption mechanisms at a concentration of about 1×10^{-3} N. Other divalent cations, notably Mn, are also active, but Mg is largely ineffective.

When Ca is added to solutions from which roots absorb Rb in the presence of Na, the impairment of Rb absorption by the Na present is only partially reversed, and only after a time lag of some minutes.

It is concluded that transport of K-Rb is through two carrier sites, one inhibited by Na, the other largely indifferent to this ion. Transport of Na also in-

volves two carrier sites, one of which is largely indifferent to K-Rb. Calcium is essential to the integrity of the selective ion transport mechanisms.

Acknowledgments

I want to thank Dr. Roy Overstreet for making available to me a quantity of Na^{22}. The barley seed was obtained from the Agronomy Department, through the courtesy of Dr. Charles W. Schaller.

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EFFECTS OF SALTS ON DPNH OXIDASE ACTIVITY & STRUCTURE OF SWEET POTATO MITOCHONDRIA [1, 2]

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The fact that inorganic salts can stimulate the oxygen consumption of many plant tissues has raised the possibility that the salts may exert a direct influence on the activity of the respiratory chain. Work in several laboratories (1, 17) has shown that the cytochrome c oxidase activity of isolated mitochondria can be stimulated by relatively high salt concentrations, but the physiological significance of this effect is not clear. Extramitochondrial cytochrome c is not the normal substrate for this enzyme, and the stimulatory effect is not specific for salts—a variety of agents which influence particle structure can promote this reaction (20). Honda, Robertson, and Gregory (9) have reported that the oxidation of reduced diphosphopyridine nucleotide (DPNH) by isolated beet root mitochondria is stimulated up to 50 % by low concentrations of salts, and they suggest that this effect may be responsible for the salt respiration of intact tissues. However, it is questionable whether or not extramitochondrial DPNH represents a physiological substrate, and further evidence on the mechanism of this effect is needed. In the course of an investigation of factors influencing the respiratory chain in sweet potato mitochondria, it was discovered that the DPNH oxidase activity is stimulated dramatically by a variety of salts. These effects are examined here, with special reference to some related salt-induced modifications of the particle structure. Preliminary reports on this work have already appeared (6, 7).

MATERIALS & METHODS

Sweet potatoes (Ipomea batatas L.) were purchased weekly at local markets and stored at room temperature. A method similar to that developed by Lieberman and Biale (15) was used for the isolation of the particulate fraction (mitochondria). Chilled tissue, 300 g, was passed through a meat grinder and then macerated at low speed in a Waring Blendor for 2 minutes with 300 ml of medium containing 0.5 M sucrose, 0.05 M tris (tris hydroxymethylaminomethane), 0.01 M ethylenediaminetetraacetic acid (EDTA), and 0.01 M cysteine-HCl, all at pH 7.0. The homogenate was centrifuged at 1,000 $\times$ g for 5 minutes, the residue discarded, and the supernatant centrifuged at 10,000 $\times$ g for 20 minutes. The pellet was resuspended in 20 ml of the isolation medium (minus cysteine), the particles were resedimented at 10,000 $\times$ g, and the final residue was suspended in 5.0 ml of 0.5 M sucrose-0.05 M tris (pH 7.0) to give the washed mitochondrial fraction (M_W). All operations were carried out at roughly 0° C. The protein content of the mitochondrial suspension, determined by the method of Lowry et al (16), varied between 2 and 8 mg of protein per ml.

Particles which were to be treated with digitonin were washed a second time in 20 ml of 0.5 M sucrose-0.005 M tris (pH 7.0) medium, resedimented, and then suspended in 8 ml of cold 1 % digitonin (Fisher) solution with the aid of a homogenizer. After standing in an ice bath for approximately ten minutes, the suspension was centrifuged at 80,000 $\times$ g for 25 to 30 minutes, and the supernatant decanted from the orange pellet. The digitonin supernatant (DS) was generally used as such; the pellet was suspended in a small volume of wash medium. In some experiments, the DS was subsequently centrifuged at higher forces to give a final supernatant and pellet.

The oxidation of DPNH was followed at 340 mμ using either a Beckman DU or Cary Model 14 spectrophotometer. Cytochrome c oxidation and reduction were measured at 550 mμ, and reduction of 2,6-dichlorophenolindophenol was followed at 600 mμ. Unless indicated otherwise, the DPNH oxidase assay mixture contained 5×10^{-5} M DPNH, 0.4 M sucrose, and 0.05 ml M_W in 3.0 ml of solution.

RESULTS

GENERAL PROPERTIES OF DPNH OXIDASE: Isolated sweet potato mitochondria can oxidize the Krebs cycle acids readily and carry out an efficient oxidative phosphorylation (8, 15). These particles will also oxidize DPNH, under the appropriate conditions, at a rate which is comparable to the rate of citrate oxidation (5–10 μatoms O_2 per hr per mg protein). The oxidation of DPNH is inhibited between 80 and 100 % by the usual respiratory chain inhibitors, including 10^{-3} M cyanide, 10^{-5} M 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), and 1 γ/ml antimycin A. Thus, both quantitatively and qualitatively, the pathways involved in the oxidation of DPNH and organic acids are similar. On the other hand, the P/O ratio, obtained by previously described methods (8), was lower for DPNH (1.4) than for citrate (2.0–3.0).

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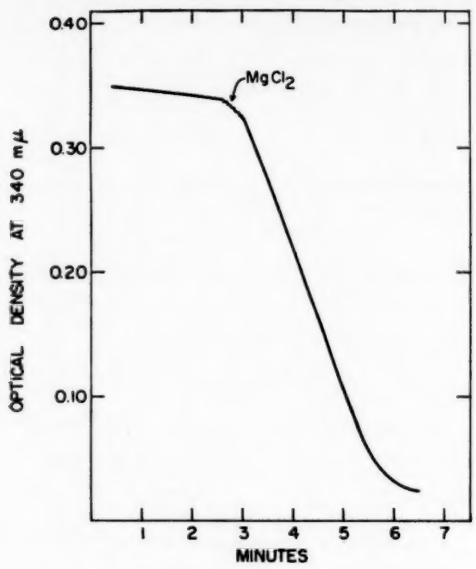

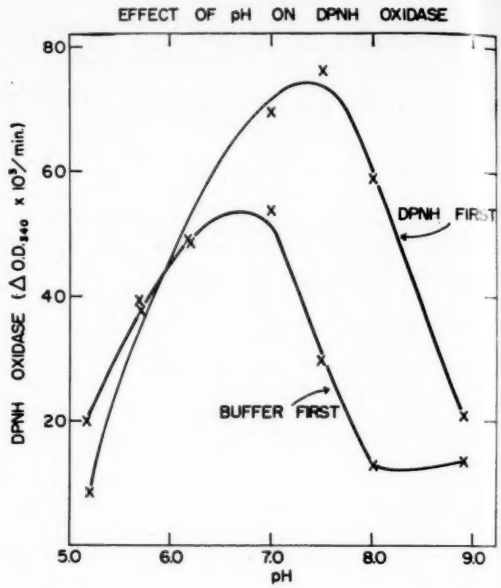

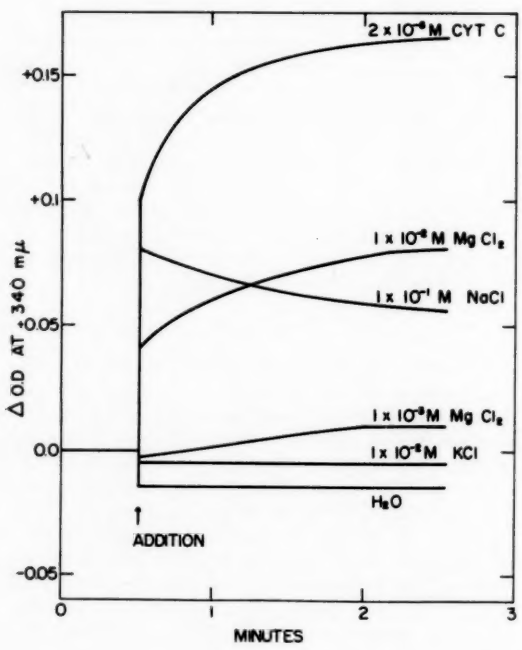

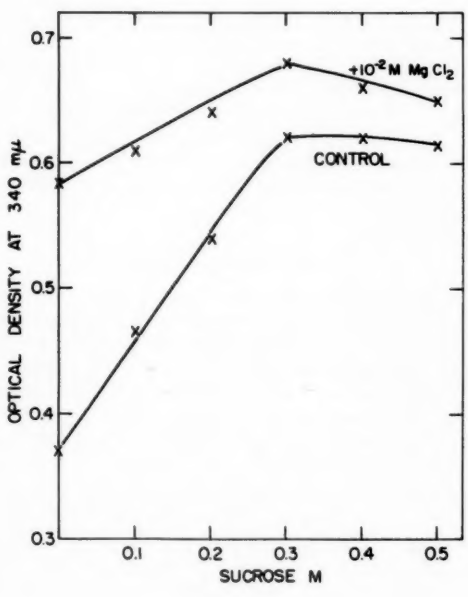

FIG. 1 (*upper left*). The effect of $MgCl_2$ on the DPNH oxidase. Reaction mixture contained 0.4 M sucrose, 5×10^{-5} M DPNH, and 0.05 ml M_W in 2.9 ml; 0.1 ml of 0.03 M $MgCl_2$ added.

FIG. 2 (*upper right*). The effect of pH on DPNH oxidase. Reaction mixture contained 0.4 M sucrose, 5×10^{-5} M DPNH, 0.01 M tris-acetate buffer, and 0.05 ml M_W in 3.0 ml. Lower curve, reaction started by adding DPNH to complete mixture; upper curve, buffer added after DPNH.

TABLE I

EFFECTS OF DIVALENT CATION SALTS ON DPNH OXIDASE

SALT	$\triangle OD_{340} \times 10^3$/min	
	10^{-4} M	10^{-3} M
O	10	10
$MgCl_2$	44	63
$BaCl_2$	41	65
$CaCl_2$	62	103
$MnCl_2$	80	111

EFFECTS OF SALTS IN UNBUFFERED SUCROSE MEDIUM: When DPNH is added to particles suspended in 0.4 M sucrose solution, it is only slowly oxidized. If a low concentration of $MgCl_2$ is then added to the mixture, the DPNH is rapidly oxidized to completion at a rate which represents as much as a tenfold stimulation (fig 1). Tests with other salts revealed that this is not a specific Mg^{++} effect, and Ca^{++} salts caused even greater stimulations (table I). The maximum effects were obtained at concentrations ranging from 10^{-3} to 10^{-2} M, with higher concentrations causing marked inhibitions. The effect is not specific for the anion, since the chloride and sulfate salts of these divalent cations cause comparable stimulations.

TABLE II

COMPARISON OF EFFECTS OF KCl & $MgCl_2$ ON DPNH OXIDASE

CONC (M)	$\triangle OD_{340} \times 10^3$/min	
	KCl	$MgCl_2$
O	10	10
10^{-4}	11	44
10^{-3}	20	63
10^{-2}	37	100
10^{-1}	54	83

Subsequent experiments showed that the salts of monovalent cations also stimulate the DPNH oxidase, although the maximum rates were somewhat lower and the salt concentrations required roughly tenfold greater. In table II, the effects of KCl and $MgCl_2$ are compared, over a wide concentration range; the differences in the effectiveness of equimolar concentrations of these salts are not due to ionic strength alone. A variety of other monovalent cation salts was tested (results shown in table III). It is clear that Na^+, K^+, and Li^+ are equally effective. Although the various univalent anions are comparable, the $SO_4^=$ salts, at equivalent molar concentrations, cause considerably greater stimulations. This suggests that the effectiveness of the monovalent cation salts is a function of the ionic strength of the medium.

TABLE III

EFFECTS OF MONOVALENT CATION SALTS ON DPNH OXIDASE

SALT	$\triangle OD_{340} \times 10^3$/min	
	10^{-2} M	10^{-1} M
O	14	14
KCl	37	54
NaCl	23	58
LiCl	25	50
KI	38	48
NaBr	23	40
KNO_3	42	53
$NaNO_3$	23	47
K_2SO_4	50	83
Na_2SO_4	58	90
Li_2SO_4	43	95

EFFECTS OF BUFFERS IN SUCROSE MEDIUM: As would be expected from these salt effects, the inclusion of buffer (pH 7.0) in the reaction mixture stimulates the DPNH oxidase (table IV). This buffer effect resembles that caused by monovalent cation salts in degree of stimulation, concentration dependence, and specificity. The pH dependence of the effect was determined, using a series of 0.01 M tris-acetate buffers. The DPNH oxidase activity was greatest between pH 7.0 and 8.0 (fig 2), but the shape of the pH-activity curve depended on the sequence in which buffer and DPNH were added to the reaction mixture. At pH's greater than 7.0, the reaction proceeded much

TABLE IV

EFFECTS OF 10^{-2} M BUFFERS (pH 7) ON DPNH OXIDASE

BUFFER	$\triangle OD_{340} \times 10^3$/min
O	9
Na-, K-Phosphate	55
Tris-chloride	50
Tris-acetate	43

FIG. 3 (*lower left*). Changes in the optical density (340 mμ) of particles (0.05 ml M_W) suspended in 0.4 M sucrose (2.8 ml), on addition of 0.1 ml of salts or cytochrome c (minus pigment absorption) to give final concentrations indicated.

FIG. 4 (*lower right*). The effect of sucrose concentration on the optical density of particle suspensions (0.05 ml M_W in 2.8 ml medium). The OD was recorded, 0.1 ml of 0.3 M $MgCl_2$ added, and the final OD measured again.

more rapidly when the particles were exposed to DPNH before the addition of buffer; this discrepancy did not appear at lower pH's. These results suggest that the access of DPNH to the reactive sites is somehow inhibited at the higher pH's. This view is supported by the finding that the omission of sucrose from the reaction mixture virtually eliminates the dependence on the sequence of addition. Using a pH 7.25 phosphate buffer-sucrose medium, the rates of DPNH oxidation ($\triangle$ OD $\times$ 10^3/min) were 15 and 80, depending on whether the buffer or DPNH was added first; when sucrose was omitted, these rates were 46 and 53, respectively. Likewise, the inclusion of 10^{-2} M $MgCl_2$ in the reaction mixture eliminates the inhibitory effect of pre-exposure to pH 7.5 buffer, presumably by altering the accessibility of DPNH to the reactive sites.

TABLE V

EFFECT OF 0.01 M TRIS-CHLORIDE (pH 6.8) BUFFER ON DIVALENT CATION STIMULATION OF DPNH OXIDASE

SALT (10^{-2} M)	$\triangle OD_{340} \times 10^3$/min	
	− BUFFER	+ BUFFER
O	10	28
$MgCl_2$	125	105
$CaCl_2$	88	125
$MnCl_2$	74	70

Since the effects of various salts on the DPNH oxidase were studied in unbuffered media, it was important to determine if they could be attributed to changes in the pH. Adding salts of the univalent cations did not significantly influence the pH of the unbuffered medium. Adding 10^{-2} M $MgCl_2$ or $MnCl_2$ lowered the pH roughly one unit. However, this pH change is not responsible for the stimulation of DPNH oxidase by divalent cation salts, since the effect can also be demonstrated when the medium is buffered at pH 6.8 (table V).

EFFECT OF TONICITY OF MEDIUM: It has been known for some time that the rate at which liver mitochondria oxidize externally added DPNH, is increased by exposure to a hypotonic medium (13); similar observations have been made with plant mitochondria (9, 26). In the present experiments, omission of sucrose from the reaction mixture caused some increase (50–100%) in DPNH oxidase activity, but this effect was only a fraction of that caused by the optimal salt concentrations (table VI). The relationship between the salt effect and the tonicity of the medium was examined. In the presence of either 10^{-3} M $MgCl_2$ or 10^{-2} M K_2SO_4, the DPNH oxidase is essentially independent of the tonicity from 0.0 to 0.4 M sucrose. On the other hand, the sucrose concentration has a marked effect when 10^{-2} M $MgCl_2$ is present: the DPNH oxidase activity is five times greater in the presence of 0.4 M sucrose than in its absence, and then declines above 0.6 M. Similar results were obtained with mannitol. This dependence of activity on the tonicity of the medium suggests that physical or structural factors play an important role.

TABLE VI

EFFECT OF 0.4 M SUCROSE ON DPNH OXIDASE IN PRESENCE & ABSENCE OF SALTS

SALT	$\triangle OD_{340} \times 10^3$/min	
	− SUCROSE	+ SUCROSE
O	20	13
10^{-3} M $MgCl_2$	90	70
10^{-2} M $MgCl_2$	35	153
10^{-2} M K_2SO_4	65	63

EFFECTS OF ADDED ELECTRON ACCEPTORS: If either ferricyanide or 2,6-dichlorophenolindophenol (DIP) is added to the reaction mixture, DPNH is readily oxidized by the particles even in the absence of added salts (table VII). This effect is most striking when sucrose is omitted. The rate of DPNH oxidation is dependent on the concentration of the electron acceptor added; the maximum effect was obtained with ferricyanide at 3×10^{-4} M. Unlike the DPNH oxidase, this reaction in the presence of dyes is completely insensitive to antimycin A and is not inhibited by exposure of the particles to pH 7.5 tris-acetate buffer. This raises the possibility that the initial steps in the DPNH oxidase and DPNH diaphorase reactions are catalyzed by two distinct flavoproteins which differ in their accessibility to external DPNH. Although DPNH is readily oxidized by these electron acceptors in the absence of salts, the reaction can be further stimulated by salts of monovalent and divalent cations. Table VIII shows this effect on the diaphorase reaction, as measured by the rate of reduction of 2,6-dichlorophenolindophenol. From this, it is clear that salts can act in the very first stage of the DPNH oxidation reaction.

TABLE VII

EFFECTS OF ELECTRON ACCEPTORS ON RATE OF DPNH OXIDATION IN ABSENCE OF ADDED SALTS

ACCEPTOR	$\triangle OD_{340} \times 10^3$/min	
	− SUCROSE	+ SUCROSE
O_2	20	15
DIP	183	80
Ferricyanide	320	120
Cytochrome c	27	55

Reaction mixtures contained 5×10^{-5} M DPNH, 0.05 ml M_W*, and, where indicated, 0.4 M sucrose, 3×10^{-5} M 2,6-dichlorophenolindophenol, 1×10^{-4} M ferricyanide, 2×10^{-6} M cytochrome c.

* Washed mitochondrial fraction.

TABLE VIII

EFFECTS OF SALTS ON DIAPHORASE REACTION

SALT	△ OD_{600} × 10^3/min	
	− SUCROSE	+ SUCROSE
O	170	38
10^{-2} M $MgCl_2$	345	162
10^{-2} M K_2SO_4	330	162

Reaction mixtures contained 5 × 10^{-5} M DPNH, 1 × 10^{-5} M 2,6-dichlorophenolindophenol, 0.05 ml M_W, and, where indicated, 0.4 M sucrose.

Cytochrome c also promotes the rate of DPNH oxidation in the absence of added salts (table VII); maximum effects are observed at concentrations of 2 × 10^{-6} M and higher. The cytochrome c-stimulated reaction differs in a number of respects from the diaphorase reaction: A, the absolute rates are considerably lower; B, the rates are greater in the presence than in the absence of 0.4 M sucrose; C, the reaction is inhibited 80 to 90 % by 1 γ/ml antimycin A and 1 × 10^{-4} M HOQNO; D, the stimulation is prevented when the particles are exposed to pH 7.5 buffer before adding the DPNH. The characteristics of the DPNH-cytochrome c reaction resemble those of the DPNH oxidase, and it seems likely that common steps are involved in these two systems.

EVIDENCE FOR STRUCTURAL CHANGES: In the course of these experiments it was noticed that the optical density (340 mμ) of the particle suspension alone increased when certain reagents were added. As shown in figure 3, these effects were studied by adding 0.1 ml of solution to 2.8 ml of a suspension of particles in 0.4 M sucrose and immediately thereafter recording the changes in OD. The OD decreases slightly when water is added, presumably due to the dilution of the suspension. The addition of low concentrations of salts prevents this dilution effect, while higher concentrations cause an actual increase in the OD. With the salts of monovalent cations, the immediate increase is followed by a slow decline in the OD; with the divalent cation salts, the initial increase is followed by a further prolonged increase in the OD. As in the stimulation of DPNH oxidase, divalent cations are effective at considerably lower concentrations than monovalent cations; for example, even at 10^{-4} M $CaCl_2$ an increase in OD can be detected. Adding low concentrations of cytochrome c also causes a striking and prolonged increase in the OD at 340 mμ, over and above the additional absorption due to the pigment itself (fig 3).

Preliminary experiments on the nature of these effects showed that the magnitude is a function of the wave length; the change in OD increases as the wave length is lowered (from 600 to 300 mμ) in a manner which suggests changes in light-scattering. Such changes have been used to investigate the morphological state of isolated mitochondria (e.g., 10), and an effort was made to determine if the observed OD changes are in fact related to structural modifications. If this were true, the OD (340 mμ) of the particle suspension should be dependent on the tonicity of the medium. The OD does increase to a maximum as the sucrose concentration in the suspension is increased to 0.3 M (fig 4); the OD remains at this level up to 0.6 M sucrose and then declines at higher concentrations. The lower OD's in hypotonic solutions could be due to a greater degree of swelling. The changes in structure must be very rapid since the OD reading remains essentially constant at the new level when particles are transferred from the original 0.5 M sucrose suspension to the various hypotonic media (fig 4). However, it is possible to detect rapid changes in the OD when particles are transferred to a medium containing a high concentration (0.8 M) of glycerol (fig 5). There is an initial rapid decrease in the OD which levels off, after 3 minutes, at the level shown by particles in water. These observations can be attributed to a rapid entrance of glycerol into the particles until the glycerol concentration is equal on both sides of a limiting membrane. The results indicate that the particles show some of the properties of osmometers, although the method employed here does not permit a critical evaluation of the structural changes. Figure 5 also shows that the initial OD of particles suspended in

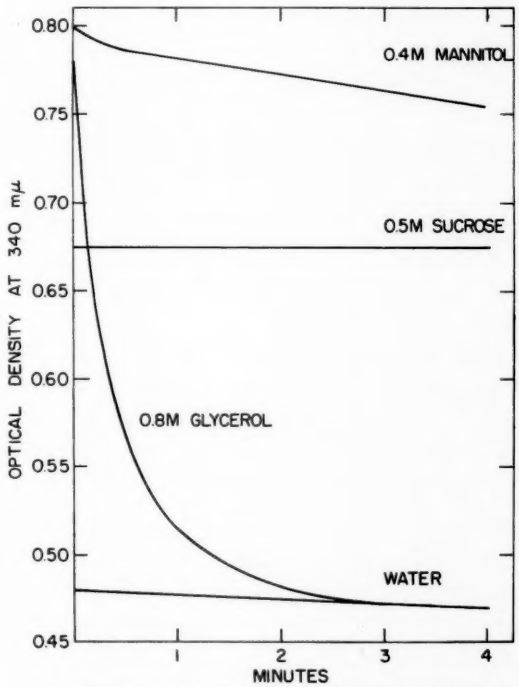

FIG. 5. Changes in the OD_{340} following addition of 0.05 ml M_W to different media.

0.4 M manitol is surprisingly high, but it declines slowly with time.

The magnitude of the $MgCl_2$-induced increase in OD is a function of the tonicity of the medium in which the particles are suspended (fig 4). The same was found to be true for the cytochrome c effect. Another variable which influences the OD is the pH of the particle suspension. The addition of a small volume of tris-acetate buffer of pH 5.0 or lower to particles suspended in 0.4 M sucrose causes a sustained increase in the OD, and the particles appear to be visibly clumped or aggregated. When the pH is above 7.0, the OD decreases markedly for several minutes. Figure 6 shows the changes in OD caused by buffers of pH 6.0, 6.8, and 7.5, and it is clear that there is relatively little effect around neutrality. From this, we can conclude that the increases in OD caused by monovalent cation salts and cytochrome c, which do not significantly alter the pH of the unbuffered suspension, are not due to changes in pH. The addition of 10^{-2} M $MgCl_2$ lowers the pH of the unbuffered sucrose medium roughly 1.0 pH unit, but this is not enough to account for the marked increase in OD. A $MgCl_2$-induced increase in the OD can be demonstrated when the medium is buffered at pH 6.0, 6.8, or 7.5, but it is smaller in magnitude at the higher pH's (fig 6). In summary, the evidence suggests that the salt-induced changes in OD are due, at least in part, to changes in the structure of the particles.

STUDIES WITH DIGITONIN FRAGMENTS: The above results suggest that the stimulation of DPNH oxidase by salts is closely related to changes in the structure of the particles. This possibility was tested further by examining some properties of the mitochondrial fragments prepared by disrupting the particles with digitonin. The relatively clear digitonin supernatant (DS) catalyzes the oxidation of DPNH, although the specific activity, on a protein basis, is considerably lower than that of the intact particles (table IX). This activity is associated with small fragments which can be sedimented (80,000 $\times$ g) after the pH is lowered to 5.0. The oxidation requires molecular oxygen and involves the whole respiratory chain, since antimycin A and HOQNO inhibit 60 to 80 % of the activity. Direct spectrophotometric examination of the DS shows that DPNH reduces the main respiratory pigments, including cytochromes of type a, b, and c. The DPNH oxidase activity of the DS is stimulated two- to threefold by added cytochrome c, and the specific activity of the cytochrome c oxidase is 50 times that of the DPNH oxidase.

The influence of salts on the DPNH oxidase activity of these fragments in the DS was then examined. In contrast to the situation with intact particles, KCl had essentially no effect (table X). Similarly, 0.01 M NaCl and 0.01 M tris buffers (pH 6.5, 7.0, 7.5) did not cause any significant stimulation. The salts of divalent cations, such as $MgCl_2$ and $MnCl_2$, did cause some stimulation (up to 50 %), but this effect was always much smaller than that seen with intact mitochondria (table X).

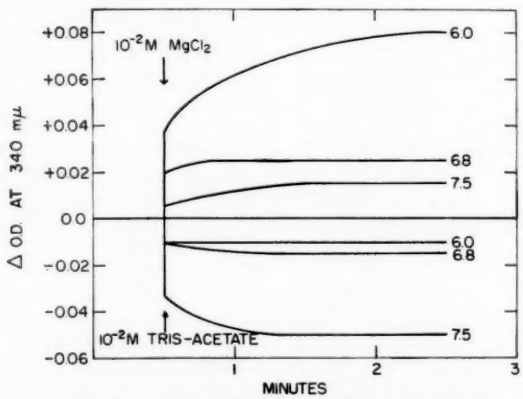

FIG. 6. The effect of pH on the OD_{340} of particles suspended in 0.4 M sucrose. Lower curves, tris-acetate buffers (final concentration = 10^{-2} M) added to unbuffered suspension; upper curves, $MgCl_2$ (final concentration = 10^{-2} M) added to suspensions already buffered at different pH's with tris-acetate.

TABLE IX

EFFECTS OF DIGITONIN TREATMENT ON DISTRIBUTION OF PROTEIN & DPNH OXIDASE ACTIVITY

FRACTION	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (Δ OD_{340} $\times$ 10^3/min $\times$ mg)
Intact mitochondria	16.8	0.13
Digitonin fractions		
Residue*	7.0	0.12
Supernatant	10.5	0.03

* Sedimented at 80,000 $\times$ g for 30 minutes.

TABLE X

EFFECTS OF SALTS ON DPNH OXIDASE OF DIGITONIN SUPERNATANT*

SALT	Δ OD_{340} $\times$ 10^3/min
O	11
0.01 M KCl	12
0.1 M KCl	9
0.001 M $MgCl_2$	15
0.01 M $MgCl_2$	7

* 0.5 ml DS in 3.0 ml assay medium.

DISCUSSION

This study has shown that the DPNH oxidase

activity of isolated sweet potato mitochondria is greatly increased by low concentrations of divalent cation salts and somewhat higher concentrations of monovalent cation salts. The divalent cation effect is not restricted to a single ion, and in this respect it is different from the Mn^{++}-stimulated DPNH or TPNH oxidases of other plant fractions (11, 23). The various buffer salts stimulate activity; no phosphate inhibition of the type observed with cauliflower mitochondria (4) was seen. The effects do appear to resemble the salt-stimulations of mitochondrial DPNH oxidase reported by Honda et al (9), although it is difficult to compare their 50 % stimulations with the tenfold increases observed here. This difference is probably due in part to their use of more complex control reaction mixtures, and this may also explain their inability to distinguish a specific divalent cation stimulation from the general ionic strength effect.

What is the mechanism by which salts stimulate the oxidation of DPNH by the respiratory chain? The results suggest that modification of the physical structure of the isolated mitochondria is an important factor in this effect. Those salt concentrations which promote activity most effectively also cause striking increases in the OD of the suspensions, and the evidence indicates that the latter effect reflects changes in particle structure. Although the present results do not permit a detailed description of these changes, an increase in OD would appear to suggest a shrinkage or contraction of the particles. In the case of monovalent cation salts, the initial increase in OD could be an osmotic effect, which would result in the movement of water out of the particles; it is followed by a decrease in OD, possibly due to the entrance of ions and water. The magnitude and time course of the OD changes caused by lower concentrations of divalent cation salts suggest that a different mechanism is involved here, and the bigger changes in structure are paralleled by greater effects on activity. The effects of 10^{-2} M $MgCl_2$ on both DPNH oxidase activity and OD of the suspension are markedly influenced by the tonicity of the medium. Finally, the absence of any marked effects of salts on the activity of the mitochondrial fragments argues for the importance of the particle structure in this phenomenon.

The structural changes could result in a greater DPNH oxidase activity if they facilitate the access of DPNH to the reactive sites of the respiratory chain. It is generally assumed that swelling of the mitochondria (decrease in light scattering) leads to an increase in permeability to DPNH, and exposure to hypotonic media does cause some stimulation of DPNH oxidase. Nevertheless, it is possible that the salt-induced changes in structure, which appear from the OD data as an apparent shrinkage or contraction, are also accompanied by an increase in permeability to DPNH. It is well known that Ca^{++} causes striking changes in the permeability of mitochondria (3, 21), and Mg^{++} may alter the particle surface (12). Monovalent cation salts can also influence the accessibility of substrates to mitochondrial enzymes (25). The finding that the access of DPNH to the reaction sites is influenced by the pH of the sucrose-containing medium suggests the properties of the particle surface are important, and these may well be influenced by salts.

The fact that DPNH is rapidly oxidized by the particles in the absence of salts if ferricyanide or DIP is present would appear to indicate that DPNH does have ready access to the oxidative enzymes. However, the flavoproteins which catalyze these diaphorase reactions may be quite distinct from the DPNH dehydrogenase which serves as the initial member of the respiratory chain, and the two types of enzyme may differ in their intramitochondrial localization. This view is supported by the differences in response of the diaphorase and oxidase to the presence of sucrose and pH 7.5 buffer in the reaction medium. DPNH may be readily oxidized in the absence of salts by an external flavoprotein which can be oxidized by dyes but is not linked to the respiratory chain. Recent studies with both isolated mitochondria and intact cells suggest that DPNH generated in the cytoplasm is not itself directly oxidized by the respiratory chain (see 2, 5). In this case, the salt-induced changes in structure of the isolated particles which permit them to oxidize external DPNH by the respiratory chain may have little relation to the tissue salt respiration.

Other explanations of the salt-stimulation of DPNH oxidase are possible. One or more reactions in the DPNH oxidase system may be accelerated by salts, and since many enzymes are influenced by the ionic strength of the medium and the presence of divalent cations, this would not be surprising. The concentrations of salts required to stimulate the cytochrome c oxidase activity of isolated plant mitochondria are relatively high (9, 17), and the effect was not seen with solubilized preparations of the enzyme (18). The primary action may therefore be on the particle structure, although a stimulatory effect has been observed with digitonin fragments of the mitochondria (22). It is also known that the salt concentration markedly influences the activity of soluble, deoxycholate-containing preparations of beef heart cytochrome oxidase (24). Sisler and Evans (22) did not find any salt stimulation of the DPNH-cytochrome c reductase activity of tobacco root mitochondria. Since the properties and interactions of the membrane-bound components of the respiratory chain are not yet well understood, it is very difficult to localize the stimulatory effect on the basis of assays of individual segments.

It is also possible that the oxidation of external DPNH by the respiratory chain is limited by the availability of intramitochondrial hydrogen acceptors (e.g. DPN^+). The level of these acceptors might be increased directly by an external oxidant, as in the case of cytochrome c, or indirectly by salts, which may cause the breakdown of an inactive or bound form. If such a breakdown involved energy-rich intermediates, some change in particle structure might also be expected (see 14, 19).

Summary

The respiratory chain oxidation of external DPNH by particles isolated in sucrose-tris-EDTA medium from sweet potato roots is stimulated as much as tenfold by inorganic salts, at concentrations ranging from 10^{-4} to 10^{-1} M. There is a specific divalent cation effect, as well as a general ionic strength effect. The DPNH oxidase activity is greatest at pH's near neutrality, and at alkaline pH's it depends on the sequence in which buffer and substrate are added. The tonicity of the medium has little effect in the absence of salts, but it does influence the activity when some salts are added. Although DPNH is rapidly oxidized in the absence of salts if ferricyanide or a dye is present, the properties of this diaphorase reaction differ from those of the DPNH oxidase.

The salts also cause marked increases in the optical density of the particle suspensions. These effects are influenced by the tonicity and pH of the medium, and the evidence indicates that they represent alterations in the particle structure.

The DPNH oxidase activity of digitonin fragments of the mitochondria is only slightly stimulated by salts.

The results suggest that the salt-stimulation of DPNH oxidase is closely related to changes in particle structure, which may facilitate the access of substrate to the respiratory chain. Some other possible explanations are also considered.

Acknowledgments

Thanks are due to Mr. Bruce Rice and Miss Sirje Ilves for capable technical assistance.

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COMPARATIVE STUDY OF PHOTOTROPIC RESPONSE & PIGMENT CONTENT IN OAT & BARLEY COLEOPTILES [1, 2]

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The receptor pigment for phototropism in higher plants is not known with certainty (15, 16, 25). Recently, several authors (4, 8, 13) have reported that preillumination of etiolated grass seedlings greatly affects both pigment content and subsequent phototropic response of their coleoptiles. This phenomenon appeared to us to be potentially useful in assessing the relative importance of various pigments suspected of being light receptors in phototropism. We have therefore endeavored to obtain quantitative descriptions of the alteration of phototropic response and of the content of various pigments in grass coleoptiles following preillumination. In this work we have used the classical phototropic object, the oat coleoptile, as well as the coleoptiles of three strains of barley, differing greatly in their content of various pigments. The comparison between oat and barley is instructive in that oat requires pretreatment with red light for repression of the first internode and promotion of growth of the coleoptile, while in barley, the first internode is genetically repressed, and the coleoptile can develop in complete darkness. However, such completely etiolated barley coleoptiles were so insensitive as to be unsuitable for phototropic experimentation, and in the end, preirradiation had to be used for both oats and barley.

MATERIALS & METHODS

I. BIOLOGICAL MATERIAL. The experiments were performed with seedlings of oat (*Avena sativa* L. var. Clinton) and two varieties of barley (*Hordeum vulgare* L. varieties Oderbrucker & Colsess). The Oderbrucker variety of barley produces normal, potentially green seedlings, while an F_2 population of this strain of Colsess barley segregates three potentially green: one albino.

The dry, unhusked seeds were individually planted in 38 × 12 mm shell vials into which had been rolled 40 × 20 mm strips of filter paper. The seeds were held in place by the paper, embryo side toward the filter paper, just below the opening of the vials. Several hundred seeds were prepared in this way and stored dry in a cold room at 2° C. To induce germination, the dry-planted seeds were transferred into a dark room (temp. 27 ± 1° C), where distilled water (ca. 1.5 ml) was added to the vials. The strips of filter paper, in addition to holding the seeds in place, also served as wicks to supply moisture to the seeds. The vials with moist seeds were arranged in 3 × 3.5 × 21 cm lucite troughs, and the whole arrangement was kept in a moist chamber in the dark room. The seedlings were used for the experiments when they were 68 to 72 hours old.

II. PREILLUMINATION. Certain seedlings were illuminated with red or blue light prior to phototropic stimulation. The oats were preilluminated from above with red light produced by a 15 w General Electric red fluorescent tube which had previously been wrapped with three thicknesses of duPont red cellophane. This red source had an emission maximum at about 650 mμ (30) and an energy output of ca. 150 ergs cm^{-2} $second^{-1}$. Two sets of barley were preilluminated, with red and blue light, respectively. The red source was similar to that described for oats, but with the intensity reduced to ca. 3 ergs cm^{-2} $second^{-1}$. The blue source was produced by a 15 w General Electric blue fluorescent tube which had been wrapped with three thickness of duPont blue cellophane. The emission maximum of this source was 450 mμ (30), and the energy output was ca. 3 ergs cm^{-2} $second^{-1}$.

III. SOURCE OF MONOCHROMATIC LIGHT FOR PHOTOTROPIC STIMULATION. Selected regions of the spectrum were obtained with a Bausch and Lomb, 500 mm focal length monochromator equipped with a tungsten filament source. The monochromator grating was 100 × 100 mm with 1,200 lines/mm, giving a linear dispersion of 16.5 A/mm. A blue Corning filter (no. CS 5-56) with a spectral transmission centered about 450 mμ was mounted at the exit slit of the monochromator. The intensity of the emergent beam was adjusted by means of a variable resistance connected in series with the light source. The opening and closing of the monochromator exit slit was controlled by an electrically operated shutter mechanism connected to a timer. Irradiances from the monochromator were measured with a type 926 vacuum phototube connected to a high-gain DC amplifier.

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The vacuum phototube was calibrated against an Eppley thermopile which in turn had been calibrated against a standard lamp from the National Bureau of Standards. The thermopile was connected to a Liston-Becker current amplifier, and galvanometer deflections were read on a Simpson volt-ohm-milliammeter. This arrangement permitted the calculation of a set of conversion factors for the translation of phototube readings into absolute energy at each wave length.

IV. Dosage-Response Curvature Experiments. The seedlings to be phototropically excited were selected for uniformity of size over a dim green, phototropically inactive light with an emission centered about 550 mμ. This safelight, shown to be phototropically inactive in preliminary experiments, was constructed by wrapping a 15 w Sylvania green fluorescent tube in three layers of amber and three layers of green duPont cellophane. All plants in which the primary leaf had pierced the coleoptile were discarded.

The vials housing the young seedlings were arranged 5 mm apart in a row on wooden block holders. Ten seedlings with coleoptiles approximately perpendicular to the incident light from the exit slit of the monochromator were simultaneously irradiated. For the experiments with oats, a cardboard shield was interposed between the coleoptiles and the incident light so that only the upper 5 mm of the coleoptile were irradiated; for the experiments with barley the entire length of the coleoptile was irradiated.

Different total monochromatic energies were obtained by varying the time of exposure while maintaining a constant intensity. Following monochromatic irradiation, the vials were rotated through 90° to allow the curvatures to develop in the plane of the row. The curvatures were shadowgraphed after the appropriate growth period, which for oats was 90 minutes and for barley 120 minutes, each growth period including the duration of exposure to monochromatic irradiation.

The shadowgraphs were taken immediately after the growth period on Kodak photographic paper. The developed shadowgraphs were laid on a tracing table, and the curvatures were measured directly on the reverse side of the shadowgraphs by means of a protractor. The method used for measuring the curvatures was essentially that of Went (27, 29).

V. Extraction, Identification, & Measurement of Blue-absorbing Pigments of the Coleoptiles. *A. Carotenoid pigments.* The pigments whose absorption spectra best fit the action spectrum for phototropism in etiolated coleoptiles are the carotenoids and the flavins (11, 15, 16, 22, 25, 26, 28). Coleoptiles were grown in large numbers for extraction of such pigments. Seeds were sown on moist filter paper in a humid chamber. When the seedlings were 24 hours old, light pretreatments were given. At the age of 68 to 72 hours the coleoptiles were harvested with a special decapitation scissors (29) under a green safelight in the darkroom, special care was taken to avoid contamination with pieces of the primary leaf.

Completely dark-grown and red or blue light pretreated coleoptiles were harvested, and the assays were carried out on whole (2.5 cm.) coleoptiles or on 0.5 cm apical and 2.0 cm basal sections cut 0.5 cm from the tip. The pigments were extracted according to the method of Goodwin (18), using acetone as initial solvent. The acetone extracts were subsequently transferred into petroleum ether (b.p. 30 to 60° C) and chromatographed on a column of adsorption alumina (80–200 mesh).

The pigments were identified by their absorption spectra, by chromatographic analysis, and by their behavior when partitioned between petroleum ether and 90 % aqueous methanol. Quantitative determinations were made by measuring the absorbancy of the separated pigment fractions at the wavelength of maximal absorption. These values were subsequently compared with those of known pigments and calculations of the pigment content of the coleoptiles made. All absorption spectra were obtained with a Perkin-Elmer Spectracord recording spectrophotometer.

B. Flavin pigments. The total flavin content of the coleoptiles was determined spectrophotofluorometrically according to the method described by Bessey, Lowry, and Love (2). 40 ml of 10 % trichloroacetic acid (TCA) were added per gram fresh weight to the homogenized, fresh coleoptile tissue. The TCA-tissue suspension was heated at 100° C for 15 minutes in subdued artificial light in order to hydrolyze flavin adenine dinucleotide (FAD) completely to flavin mononucleotide (FMN). After hydrolysis and centrifugation, the clear supernatant containing FMN and small amounts of free riboflavin was neutralized with a fourth its volume of 4.0 M K_2HPO_4. The neutralized extracts were brought to a final volume of 200 ml/gram fresh weight by adding distilled water. At this concentration the extracts had a pH of 6.8. The flavins are known to fluoresce maximally at this pH (21).

The spectrophotofluorometer consisted of two Bausch and Lomb grating monochromators operating on a Xenon arc light source. One of the monochromators (M_1) was used to produce the excitation wavelength (370 mμ), while the other (M_2) analyzed the fluorescence at 525 mμ and other wavelengths. The intensity of the fluorescence energy was recorded by means of an amplifier connected to a sensitive photocell mounted at the exit slit of M_2. The fluorescence spectrum was directly recorded on a servo recording attachment coupled to the spectrophotofluorometer. The capacity of the fluorometer cuvette was 5.0 ml.

Blanks for the determination of zero fluorescence were prepared by adding 5.0 mg crystalline $Na_2S_2O_4$ to the samples after reading the fluorescence. This reagent reduces FMN to the non-fluorescent form.

A standard FMN solution was prepared contain-

ing 0.3 μg FMN per ml TCA-K_2HPO_4, in concentrations similar to those of the unknown samples. The FMN content of the unknown samples was calculated using the following formula:

$$\frac{\text{Fluorescence of sample-blank}}{\text{Fluorescence of standard-blank}} \times$$

$$0.3 \times \text{dilution factor} = \mu g \text{ in sample}$$

FMN was shown to yield a linear concentration-fluorescence relationship between 0.001 to 0.35 μg/ml in the TCA-K_2HPO_4 solvent.

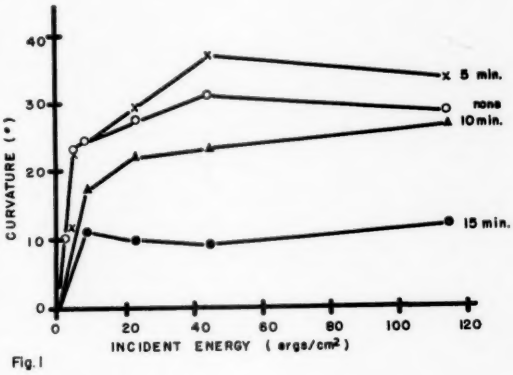
Experimental

I. Phototropic Responses of Oat Coleoptiles. In view of the fact that Clinton oats have not been previously used in quantitative experiments on phototropism, we felt it necessary to calibrate their behavior in certain preliminary experiments.

A. Dosage-response studies. Four sets of oat seedlings were grown under different regimes of red light as follows: One set was given a total red light pre-illumination of 15 minutes (15 R), delivered in three 5-minute doses at 6, 30, and 54 hours after moistening. A second set was given two 5-minute doses (10 R) 6 and 30 hours after moistening. The third set was given only one 5-minute illumination (5 R) six hours after moistening. Finally, a set of completely etiolated seedlings was kept as control. When the plants were 68 to 72 hours old they were unilaterally irradiated with progressively increasing irradiance of blue light at 460 mμ.

The data obtained from these studies are summarized in figure 1. The principal conclusion to be derived from these experiments is that red light pretreatments greatly affect the subsequent phototropic response of oat coleoptiles, for at each level of red light pretreatment, a different dosage-response curve was obtained. In figure 2 curvatures elicited by standard blue light energies at 460 mμ are expressed as a function of the red light pretreatments. At each of the three levels of blue energy plotted in the graph the relationship between phototropic sensitivity and the amount of red light pretreatments follows the same pattern. The 10-minute red pretreated plants produced smaller curvatures than either the completely etiolated controls or the 5-minute pretreated plants, and the curvature responses were decreased further as the total red pretreatment was raised to 15 minutes. There is considerable evidence that the magnitude of the red light effect depends upon the distribution of the energy in time. This consideration is important in view of the fact that at each of the red light dosages reported here, the coleoptiles compared in figure 2 were of different ages.

It appears that 5 minutes of red light at the stated intensity given 6 hours after sowing significantly enhances the phototropic response of the coleoptiles, while each additional 5-minute pretreatment given 24 hours apart during the growth of the coleoptiles decreases photosensitivity. Blaauw-Jansen (4) has recently reported that red light pretreatments enhance the phototropic responses of oat coleoptiles, while Curry et al (13) and Briggs (8) had reported such pretreatments to depress curvatures in corn coleoptiles. Our data indicate that both these results are part of the same general phenomenon.



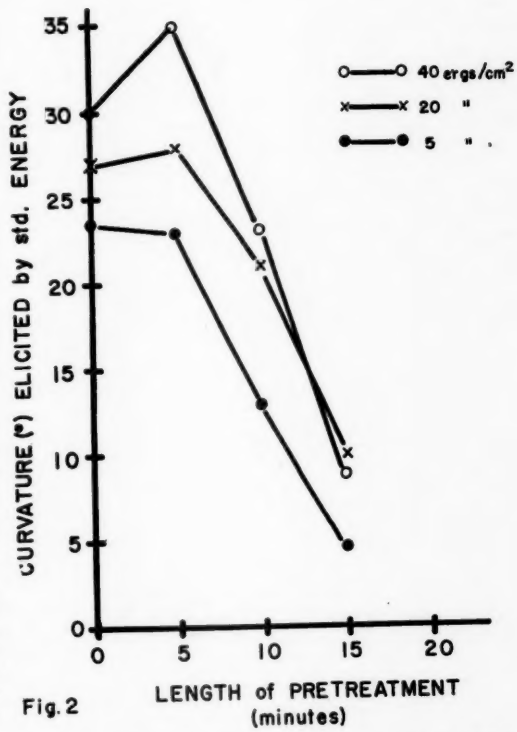


Fig. 1. Dosage-response curves for oat coleoptiles. The times indicated in the graph represent the duration of red light pretreatments.

Fig. 2. Relationship between red light pretreatments and subsequent phototropic response in oat coleoptiles. Standard excitational energies applied at 460 mμ.

B. Reciprocity test and action spectrum for phototropism. One of the conditions that must be fulfilled in a photobiological process before a valid action spectrum may be constructed is that reciprocity be obeyed, i.e. that the product of intensity × time be a constant (5, 6). As a first step in the construction of an action spectrum for the phototropic responses of Clinton oat coleoptiles, we decided to test reciprocity by the use of the 10 R plants of the previous experiment. The choice of pretreatment was dictated by the fact that plants in this condition gave a log-dosage response which was linear over a fairly wide range of energy dosages (fig 3). The intensities of blue light selected at 460 mμ for the reciprocity test were 1.0, 2.0, 4.0, 5.0, and 10.0 ergs/cm²/second. Each of these intensities of corresponding length of exposure was selected in order to produce a constant irradiance of 20 ergs/cm². This total incident energy was chosen for convenience since it had been used in previous experiments to obtain more than half maximal first positive curvatures.

Within the ranges of blue light intensity used the coleoptiles were found to obey the reciprocity law (fig 4), thus corroborating the results of Blaauw (3), and of numerous other authors.

The action spectrum for phototropism (fig 5) was constructed by plotting the number of quanta required to produce a standard positive curvature at wavelengths between 400 and 500 mμ. Each of the three action spectra plotted, for constant responses of 10, 15, and 20°, had three peaks at ca. 480, 460, and 423 mμ, the 460 mμ peak being the maximum. The action

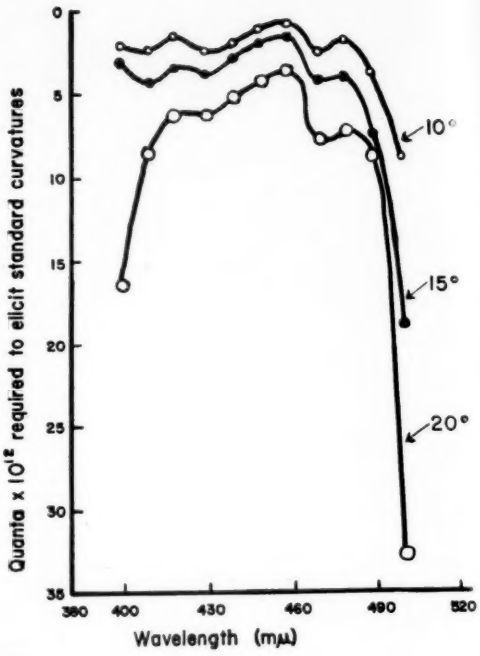


Fig. 5

FIG. 5. Action spectrum for phototropism in oat coleoptiles calculated for standard curvatures of 10°, 15°, and 20°.

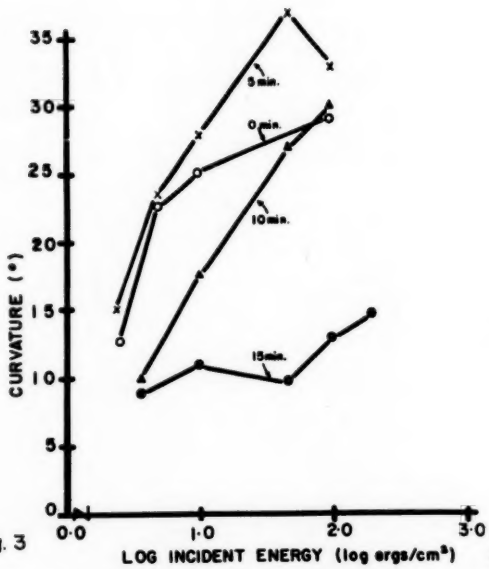


Fig. 3

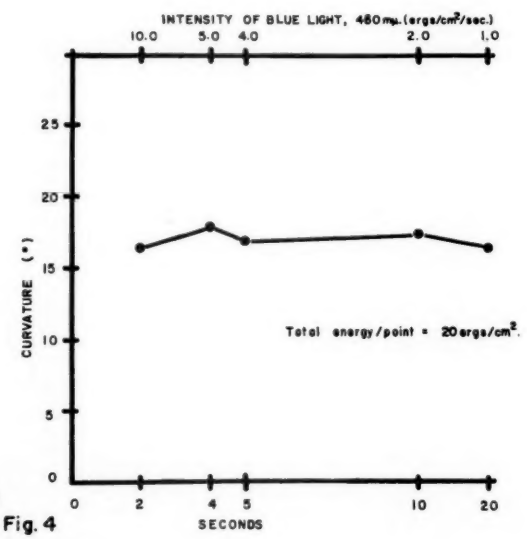


Fig. 4

FIG. 3. Log-dosage-response curves for oat coleoptiles.
FIG. 4. Reciprocity relationships for first positive oat coleoptile curvatures.

spectrum for 20° curvature closely resembles several which have recently been obtained for oat coleoptiles and for Phycomyces sporangiophores (12, 14, 23).

II. Phototropic Responses of Barley Coleoptiles (var. Oderbrucker). Several preliminary experiments were necessary in order to achieve a quantitative method for the study of phototropism in barley coleoptiles. Completely etiolated coleoptiles were grown according to the method already described. When ca. 65 to 70 hours old, the plants were exposed to the same phototropic stimuli as were given the oat coleoptiles. No curvatures developed. After several trials it became evident that prolonged (11–90 minutes) unilateral exposures to blue light were necessary in order to obtain positive phototropic curvatures with these barley coleoptiles, and that their phototropic response could be very significantly enhanced by pretreatments with red or blue light, blue light being the more effective.

A. Relationship between pretreatments & subsequent phototropic activity. The experiments were performed with coleoptiles of barley var. Oderbrucker grown either in darkness with red light pretreatment or with blue light pretreatment. The pretreatments were begun 24 hours after the seeds had been sown, and were, whether red or blue, administered continuously for 12, 24, and 42 hours. In all, seven pretreatment conditions were thus established. After each pretreatment, the plants were kept in darkness until they were 65 to 70 hours old, at which time they were used for the experiments.

For each of the seven pretreatment conditions already described a pair of dosage-response curves was obtained by using two different intensities of blue light at 450 mμ applied unilaterally. Thus, for all the seven pretreatment conditions a total of 14 dosage-response curves was obtained. These dosage-response curves are presented in figures 6a to g. For each pair of dosage-response curves belonging to the same family of pretreatment but representing different intensities of unilaterally applied blue light, the curves are parallel but do not coincide. A given total energy elicits a different magnitude of response depending on its distribution in time, and invariably, the longer and weaker irradiations produce greater curvatures than do the shorter more intense ones. This indicates that the reciprocity law is not followed in the phototropic response of barley coleoptiles.

The relationships between pretreatments and subsequent phototropic activity are plotted in figure 7. The intensity of the excitational blue energy was 2.8 ergs cm^{-2} $second^{-1}$. Each point on the upper pair

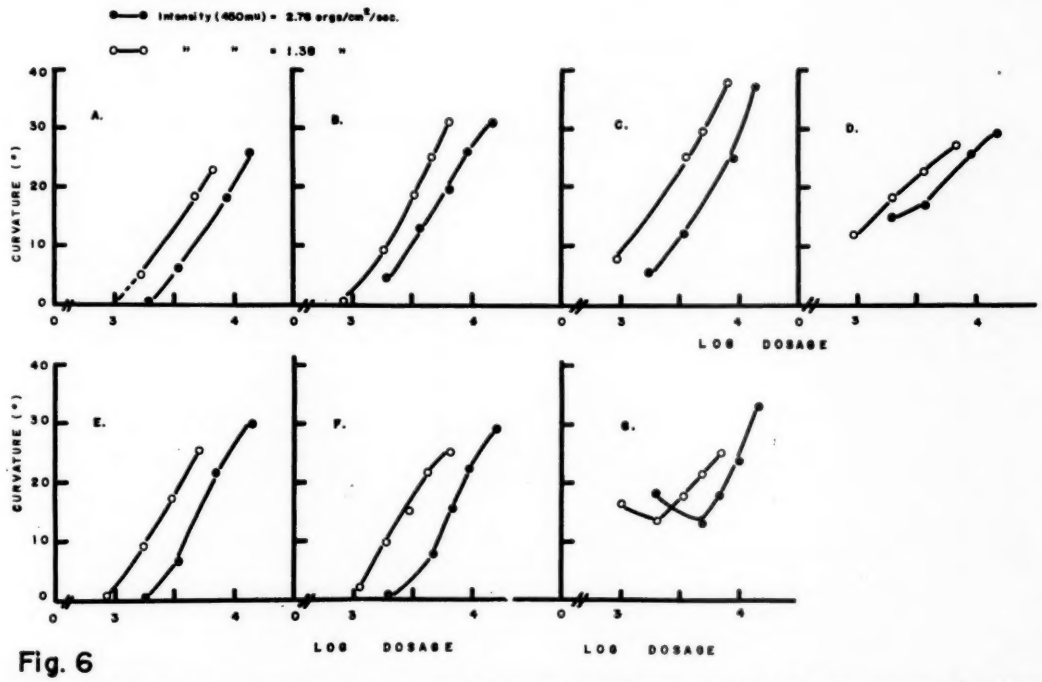


Fig. 6

Fig. 6. Log-dosage-response curves for barley grown under different conditions of pretreatment. A. Completely etiolated. B. 12 hours blue light pretreatment. C. 24 hours blue light pretreatment. D. 42 hours blue light pretreatment. E. 12 hours red light pretreatment. F. 24 hours red light pretreatment. G. 42 hours red light pretreatment. Note apparent decrease of threshold-response energy with increasing lengths of blue or red pretreatment.

of curves represents the curvature obtained after 90 minutes continuous unilateral irradiation with blue light. Points on the lower pair of curves represent curvatures obtained with 45 minutes of such continuous irradiation. It is evident that (1) the longer period of unilateral excitation with blue light yielded greater curvatures than the shorter period of excitation, (2) the preirradiated plants are more phototropically active than the non-pretreated ones, and (3) blue light pretreatment is more effective for potentiating phototropic response than is red light pretreatment.

The maximum potentiating effect of blue light was attained after 24 hours of pretreatment. Thereafter, further increase in the length of pretreatment resulted in a decreased sensitivity. By and large, the effects of red light pretreatment were less dramatic than those described for blue. However, in all cases of red light pretreatment there was a measurable increase in phototropic response over that of completely dark-grown coleoptiles.

The dosage-response curves for plants which had been pretreated with red light for 42 hours (fig 6g) were markedly different from all the others, in that there was a suggestion of a double-peaked response. The first part of the response, which reaches its lowest ebb after 22.5 minutes of unilateral blue light irradiation, is followed by a second increasing response, giving the dosage-response curve a trough-shaped appearance.

B. Test for reciprocity. Reciprocity was tested under three conditions of pretreatment: 1, Complete etiolation, 2, 6 hours of red pretreatment, 3, 6 hours of blue pretreatment. Both red and blue light pretreatments were started 24 hours after the seeds had been sown. Coleoptiles were unilaterally exposed to four different light intensities at 450 mμ, and the corresponding lengths of exposure were selected so as to obtain a constant total irradiance of ca. 7,500 ergs/cm². The phototropic responses when plotted for constant dosages at different light intensities showed a marked deviation from reciprocity (fig 8), in that plants exposed to lower intensities for a longer time showed a higher response than those which were given the same total irradiance for a shorter time. Briggs (7) has found a similar case of non-reciprocity at certain energy levels in oat and corn coleoptiles.

C. Action spectrum for phototropism in barley. The failure of reciprocity makes the construction of a valid action spectrum difficult. In the following attempt, the variability of responses introduced by non-reciprocity was minimized by choosing identical irradiation times and intensities at each of the different

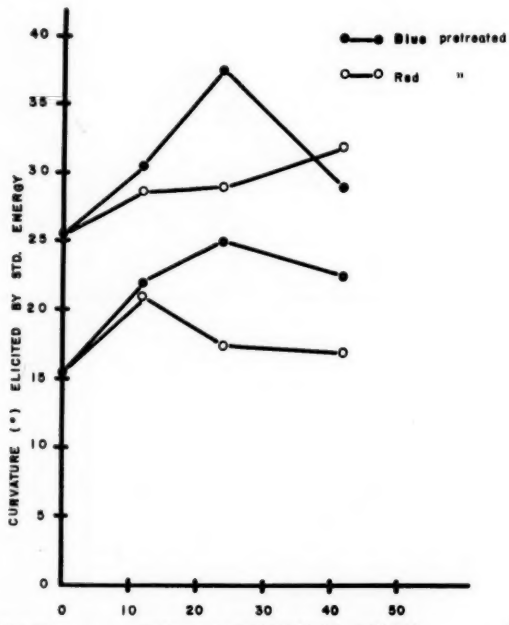


Fig. 7

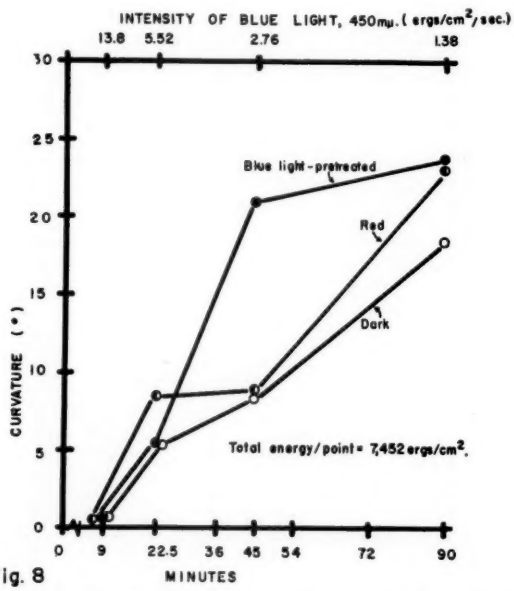


Fig. 8

FIG. 7. Effect of pretreatment of barley coleoptiles with red or blue light upon curvatures elicited by standard energies of blue light at 450 mμ. For the upper pair of curves total blue excitational energy at each point = ca. 15,000 ergs/cm², delivered at the rate of 2.8 ergs/cm²/second for 90 minutes. Points on the lower pair of curves represent total excitational energy of ca. 7,500 ergs/cm², delivered at the same rate as indicated above for 45 minutes.

FIG. 8. Reciprocity relationships for barley curvatures after different conditions of light pretreatment.

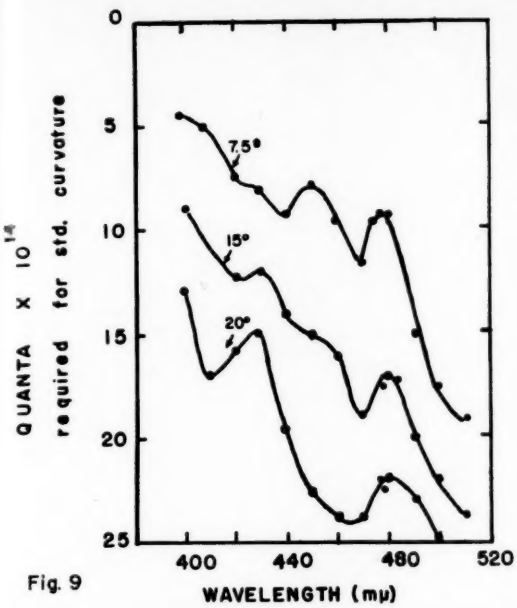


Fig. 9

FIG. 9. Action spectra for phototropic responses of barley coleoptiles, calculated for standard curvatures of 7.5°, 15°, and 20°.

wavelength values. The seedlings used in this experiment were pretreated with red light (12 ergs cm^{-2} sec^{-1}) for 45 minutes 36 hours after planting and were used for the experiment when they were 68 to 72 hours old.

Three action spectra calculated for standard curvatures 7.5°, 15°, and 20° are shown in figure 9. The standard curvatures mentioned represent continuous unilateral irradiations for periods of time ranging from 20 to 60 minutes. The action spectrum calculated for 7.5° has peaks at 480 and 450 mμ; the action spectra calculated for 15° and 20° curvatures showed the peak at 480 mμ and another peak at ca. 425 mμ. All three curves showed a trend toward continued activity into the near-ultraviolet. This action spectrum is quite different from the action spectrum for the first positive tip curvature of Avena. It resembles, however, the action spectrum for the base curvature published many years ago by Haig (19).

III. COMPARATIVE PHOTOTROPIC RESPONSES OF NORMAL & ALBINO BARLEY (VAR. COLSESS). The seedlings used in this experiment were pretreated with red light (12.0 ergs $cm^{-2}sec^{-1}$) for 45 minutes at the age of 36 hours. The subsequent phototropic excitational energy (450 mμ) was 2.8 ergs cm^{-2} $second^{-1}$ applied for 30, 70, 90, or 120 minutes. The log-dosage response curves for the two types of Colsess barley are presented in figure 10. The main facts brought out by this experiment are: A, The energy threshold for phototropic response in the potentially green barley coleoptiles (ca. 1,500–2,000 ergs/cm^2) is lower than that for the albino coleoptiles (ca. 5,000–8,000 ergs/cm^2). B, The potentially green coleoptiles produce maximum curvatures at a lower level of excitational energy (ca. 11,500 ergs/cm^2) than the albino coleoptiles (20,000 ergs/cm^2). C, At the respective energies for maximal phototropic activity, the albino coleoptiles produce larger curvatures than the potentially green coleoptiles.

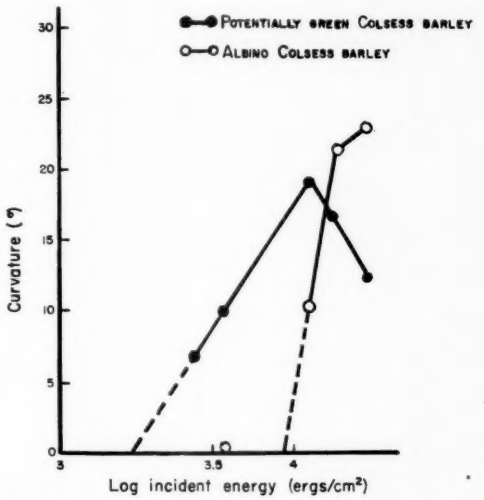


Fig. 10

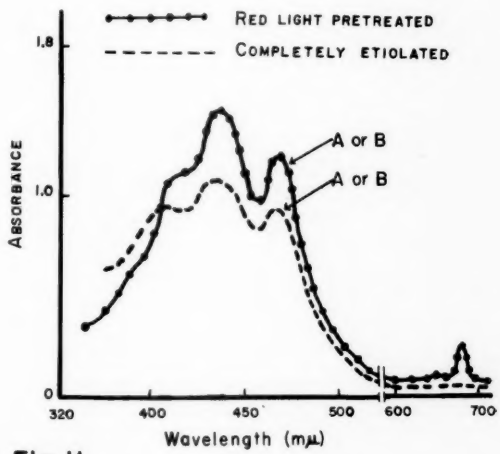


Fig. 11

FIG. 10. Log-dosage-response curves for coleoptiles of albino and potentially green barley.

FIG. 11. Gross absorption spectra of the lipid-soluble pigments of completely dark-grown and red light pretreated coleoptiles of oats (Solvent: petroleum ether). A. Pigments of the coleoptile tip. B. Pigments of the coleoptile base.

TABLE I

RELATIVE CAROTENOID CONTENT OF COMPLETELY DARK-GROWN & RED LIGHT PREILLUMINATED COLEOPTILES OF OATS & BARLEY

PLANT MATERIAL (COLEOPTILES)	PRETREATMENT (hr)	CAROTENOIDS OD AT 439 mμ IN 1 ml OF SOLVENT		
		TOTAL	PER COLEOPTILE	PER g fr wt
Oderbrucker barley	None	0.65	0.0013	0.045
Oderbrucker barley	Red for 24 hr	1.70	0.0034	0.13
Oderbrucker barley	Blue for 24 hr	1.20	0.0024	0.10
Oats	None	1.1	0.0022	0.73
Oats	Red for 10–20 min	0.60	0.0086	0.46

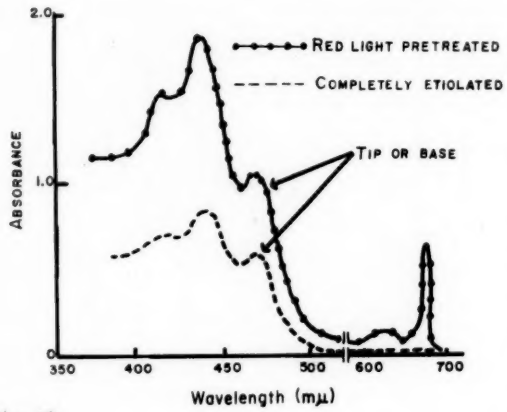


Fig. 12

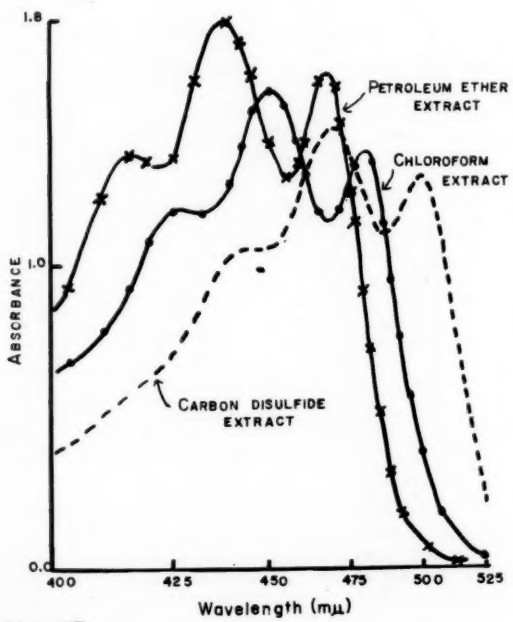


Fig. 13

On the basis of threshold energies required for the phototropic response, the order of photosensitivity of the various coleoptiles studied may be summarized as follows: Oats > Oderbrucker barley > normal Colsess barley > albino Colsess barley. The oat coleoptiles were more than 1,000 times more sensitive than all varieties of barley tested.

IV. CAROTENOID PIGMENTS OF COLEOPTILES. *A. Gross absorption spectra.* 1. Oats. Prior to chromatography, the gross absorption spectra of the total pigments in the coleoptiles were determined for the different conditions of light pretreatment (fig 11). The relative quantities of pigments are given in table I. The absolute amount of pigment in preilluminated coleoptiles of oats is higher than that of completely etiolated coleoptiles when comparison is made on the basis of amount of pigment per coleoptile. However, when pigment content is compared on a fresh weight basis, the completely etiolated oat coleoptiles appear to be richer in carotenoids than the coleoptiles pretreated with red light. This discrepancy is due to the fact that preillumination with red light causes an increase in the fresh weight of the coleoptile, this increase is relatively greater than the increase in carotenoids.

2. Barley. With either of the methods of comparison, the completely dark-grown barley coleoptiles contained less than half the amounts of pigment obtained from coleoptiles illuminated for 24 hours or more with either red or blue light. Preilluminations lasting longer than 24 hours led to a further increase in carotenoid pigments until, finally, the gross absorption spectrum was dominated by chlorophyll (fig 12).

B. Chromatographic separation of carotenoid pigments. 1. Completely etiolated coleoptiles. Lutein was the only spectrophotometrically detectable carotenoid obtained from oat and barley coleoptiles

FIG. 12. Gross absorption spectra of the lipid-soluble pigments of barley coleoptiles.

FIG. 13. Absorption spectrum in various solvents of lutein extracted from oat coleoptiles.

TABLE II

ZONAL DISTRIBUTION ON AN ALUMINA COLUMN OF CAROTENOIDS & CHLOROPHYLLS OF RED OR BLUE LIGHT PREILLUMINATED COLEOPTILES OF OATS & BARLEY

ZONE NO.	DESCRIPTION	ABSORPTION MAXIMA IN PETROLEUM ETHER	% (v/v) OF ACETONE REQD. TO ELUTE ZONE FROM COLUMN	IDENTIFICATION
I	* Orange	475, 447.5, 442 mμ	1	β-carotene
II	* Yellow traces		10	Probably xanthophyll epoxide
III	Green	664, 618, 580, 432, 410	20	Chlorophyll *a*
** IV	* Yellow	468, 440, 417, 396 ~	50	Lutein
V	* Yellow traces	Maxima in $CHCl_3$ at 478, 448, 422, 400	Eluted with MeOH	Probably neoxanthin

~ indicates inflection point.

* A mixture of zone IV and the pigment of completely dark-grown coleoptiles was chromatographically inseparable. Both pigments, as well as the pigment of zone V were hypophasic when partitioned between petroleum ether and an aqueous solution of 90 % MeOH, while the pigments of zones I, II, and III were epiphasic.

** The U.V. spectrum of zone IV in $CHCl_3$ had maxima at 335 and 322 mμ.

grown under conditions of complete etiolation. Its absorption spectrum in various solvents is shown in figure 13. The oat coleoptiles were ca. 15 times richer in this pigment than coleoptiles of barley (table I). In both oats and barley, traces of β-carotene were detectable at the appropriate position on the chromatogram, but these amounts were too minute for convenient spectral analysis.

2. Coleoptiles illuminated with red or blue light. The distribution of pigments on an alumina column, developed with petroleum ether (b.p. 40–60° C) containing varying concentrations of acetone, is shown in table II. It is clear from this table that the lipid-soluble pigments of coleoptiles illuminated with red or blue light are similar to those commonly encountered in chloroplasts (24). Although there were marked quantitative differences (table I), the pigments of oat and barley coleoptiles were found to be qualitatively identical.

3. Distribution of carotenoid pigments in tip & base of oat & barley coleoptiles. In tables III and IV are presented the distribution of carotenoids in tip and base of the coleoptiles studied. The data for oat coleoptiles (table III) show that the same types of pigments are present in the tip and base, the tip having the greater pigment content. Among the three types of barley assayed (table IV), the albino Colsess coleoptile was most deficient in carotenoids, the potentially green Colsess was intermediate, and the Oderbrucker was richest. The albino coleoptiles, however, contained no spectrophotometrically detectable amounts of chlorophyll. In all the coleoptiles assayed, the predominant carotenoid was a xanthophyll (more than 85 %).

The report by Bünning (9) that the oat coleoptile tip contains larger amounts of carotenoids than the base is thus corroborated in the present investigations. However, Bünning's recent (10) proposal that pigments of the coleoptile tip are different from those contained in the base is not supported by our data.

TABLE III

DISTRIBUTION OF CAROTENOID PIGMENTS IN TIP & BASE OF RED LIGHT PRETREATED OAT COLEOPTILES

PIGMENT	AMOUNT (μg)/g fr wt	
	TIP	BASE
Total carotenoids	3.5 μg	1.1 μg
β-Carotene	0.6	0.17
Xanthophyll (Lutein)	2.9	0.93

TABLE IV

DISTRIBUTION OF CAROTENOID PIGMENTS IN TIP & BASE OF RED LIGHT-PRETREATED BARLEY COLEOPTILES

PLANT MATERIAL	TOTAL CAROTENOIDS AMOUNT (μg)/g fr wt		RATIO OF PIGMENT IN TIP : BASE
	TIP	BASE	
Albino Colsess barley	0.70	0.27	2.6
Potentially green Colsess barley	1.8	0.33	5.5
Oderbrucker barley	2.4	0.49	5.0

V. FLAVIN PIGMENTS. The flavin content of oat and barley coleoptiles under various conditions of light pretreatment is given in table V. The figures show that FMN is higher in oat coleoptiles than in barley coleoptiles, while normal and albino barley coleoptiles appear to contain the same amounts of FMN. In all coleoptiles assayed, FMN appears to be most concentrated in the tip, and illumination of the coleoptiles with red or blue light caused an increase in FMN content. The flavin contents reported in table V are considerably higher than those reported by Galston and Baker (17), who used a microbiologi-

TABLE V

FMN CONTENT OF OAT & BARLEY COLEOPTILES

PLANT MATERIAL	PRETREATMENT	SECTION	μg FMN/g fr wt
Oat coleoptile	Completely etiolated	Whole	24.0
"	10 min of red	Tip	56.0
"	"	Base	25.0
Oderbrucker barley	Completely etiolated	Tip	16.3
"	"	Base	10.6
"	24 hr of red	Tip	26.4
"	"	Base	19.5
"	24 hr of blue	Tip	30.0
"	"	Base	21.0
Albino Colsess barley coleoptile	* Completely etiolated	Tip	27.5
"	"	Base	12.0
Normal Colsess barley coleoptile	"	Tip	30.0
"	"	Base	19.0

* Harvested in white light.

cal assay. They pointed out that their figures may have been spuriously low because of inhibitors, for at progressively greater dilutions of their samples the apparent flavin content was higher. This would indicate that the present data, based on fluorometric measurements, are the more reliable.

VI. RELATIVE ABSORBANCIES OF TOTAL FLAVINS & CAROTENOIDS IN OAT & BARLEY COLEOPTILES. From the pigment data presented in tables III, IV, and V, the relative absorbancies of the reported amounts of flavin and total carotenoids can be calculated. Such ratios would indicate the relative contribution of each pigment to the attenuation of light energy across the coleoptile, assuming that the pigments were uniformly distributed in the cell. The absorbancies of standard solutions of carotenoids (β-carotene & lutein) and FMN were determined at the wavelengths of maximal absorption. The absorbancy of 100 μg riboflavin or FMN in 1.0 ml H_2O (1 cm light path) at 450 mμ is 1.8. Similarly, the absorbancy of 5.0 μg β-carotene (at 447.5 mμ) or lutein (at 440 mμ) in 1.0 ml petroleum ether is 1.1. From these relationships the absorbancy ratios of coleoptile pigments presented in table VI were calculated.

TABLE VI

CAROTENOID: FLAVIN ABSORBANCY RATIOS AT COLEOPTILE TIP

PLANT MATERIAL	PRETREATMENT	RELATIVE CAROTENOID: FLAVIN ABSORBANCIES IN COLEOPTILE TIP
Oat	Completely etiolated	1.55
Oat	10 min of red	0.70
Oderbrucker barley	24 hr of red	1.11

It appears that the relative absorbancies vary with the pretreatment and the plant material, but that under the conditions used here, there is approximately equal light absorption by these two classes of pigments. Preirradiation, which alters subsequent phototropic sensitivity, results in a marked rise in both flavin and carotenoid pigments in the coleoptile. Because the magnitude of the flavin increase is greater than that of the carotenoid increase, the overall effect of preirradiation is to increase light absorption by flavins relative to that of carotenoids.

DISCUSSION

The pigment analyses demonstrate that the etiolated oat and barley coleoptiles have the same kinds of blue-absorbing pigments which may operate as photoreceptors in phototropism. It has not been possible in the present study to decide between flavin and carotenoid as the actual photoreceptor. This was true because the A, so-called albina barley turned out to have appreciable quantities of carotenoid pigments, and B, light pretreatments which caused an alteration of phototropic sensitivity also caused concurrent increases in both carotenoid and flavin content. The reported differences in the threshold energy requirements for phototropic curvatures in oats and in the three varieties of barley studied correlate with differences in pigment content; the more sensitive oats were richer in both the flavins and carotenoids than were all the barley varieties.

In so far as the reciprocity law is not followed by barley, the action spectrum obtained with that plant must be considered unreliable. The action spectrum obtained with Clinton oats (in which reciprocity is followed) possesses maxima which lie approximately at 480, 460, and 425 mμ, and are in reasonable agreement with those previously obtained for oats by Shropshire with Withrow (23), and for Phycomyces by

Curry and Gruen (12), and by Delbrück and Shropshire (14). It has been shown by these groups of investigators that an additional peak of phototropic activity does occur in the near-ultraviolet at approximately 360 to 370 mμ. This near-ultraviolet peak, which is characteristic of flavin absorption, cannot be identified with absorption by any carotenoid known to exist in the coleoptiles of oats, barley, or Phycomyces.

In the wavelength regions 410 to 500 mμ, the flavins and the carotenoids possess absorption spectra that are almost identical, depending upon the nature of the solvent used. For example, while FMN in water possesses only two prominent maxima at 450 mμ and 380 mμ, in aqueous pyridine it shows two *additional* peaks in the visible region, at 470 and 420 mμ, and the near-ultraviolet peak is shifted to 350 mμ. The visible spectrum of 3-methyllumiflavin in benzene (20) closely resembles that of the carotenoid lutein in organic solvents (fig 13). Also, the electron-transferring flavoprotein described by Beinert and Crane (1) shows absorption characteristics similar to those of carotenoids, with the exception of the near-ultraviolet peak, which is not shown by the carotenoids.

These facts contradict the frequent statement that flavins possess only one absorption maximum in the blue, and that the multiple-peaked action spectra for phototropism can therefore be attributed only to carotenoid absorption (12, 23). The relative absorbancy data for carotenoids and flavins presented in table VII show that the contribution of each pigment to the absorption of light within the coleoptile tends to be about the same, varying somewhat with light pretreatment and the plant material. It is thus conceivable that either pigment functions as the sole photoreceptor, or that both pigments play some role in phototropism, and that they contribute jointly to the shape of the action spectrum curve.

The phototropic responses of barley appear to differ markedly from those of oats. The most striking of these differences are: A, The responses of barley do not follow the reciprocity law; a given total excitational energy given in a longer exposure is more effective than the same energy given in a short exposure. B, Much larger total energies are required to elicit curvature. C, The action spectrum shows a continued increase in activity with decreasing wavelength of light employed. These differences could all be explained in terms of the generalization that barley lacks a first positive curvature, and that the curvature being studied here is equivalent to the second positive curvature on the Avena curve.

Briggs (7) has proposed that in plants such as oats which follow reciprocity at low light intensities of phototropic excitation, the failure of reciprocity at high excitational energies may be due to inactivation of the photoreceptive pigments. Prolongation of the excitation could lead to a regeneration of pigment and a resumption of phototropic activity. This hypothesis may also explain the situation observed in barley, with the omission of the first step involving initial reciprocity. These possibilities must be tested further.

Summary

A comparative study has been made of phototropic behavior in one strain of oats and three strains of barley including a so-called albino. The major differences revealed are A, Oats require preirradiation with red light to repress growth of the first internode, while in barley, the first internode is genetically repressed. In both types of plants preirradiation with red or blue light alters phototropic sensitivity and pigment content, as follows: In oats, low preirradiation dosages of red or blue light enhance and higher dosages inhibit phototropic response sharply; in barley all but extremely long preirradiation dosages enhance subsequent sensitivity. B, Oat coleoptiles obey the reciprocity law over a wide range of energies eliciting first positive curvature, while barley coleoptiles do not. C, Oat coleoptiles have an action spectrum with three peaks in the blue, a peak in the near ultraviolet and a deep trough near 400 mμ; barley coleoptiles seem generally to increase in sensitivity with decreasing wavelength and show no trough near 400 mμ. D, Oat coleoptiles are higher in both flavin and carotenoid pigments than are corresponding barley coleoptiles. In both oats and barley, light enhances the formation of both types of pigments. Etiolated coleoptiles of both plants have lutein as the major carotenoid; β-carotene appears only after some light exposure. E, Albino Colsess barley, which has a slightly depressed phototropic sensitivity, has lower than normal carotenoid, but approximately normal flavin content.

Coleoptile tips contain ca. three to five times as much carotenoid and two times as much flavin as the base; the pigments are qualitatively the same in both regions. Calculations of total absorbancies of the carotenoid and flavin pigments at the tip reveal them to contribute approximately equally to light attenuation across the photoreceptor region. On this basis, and also on the basis that flavins and carotenoids exhibit similar absorption curves in organic solvents, with three peaks in the visible, it is concluded that the visible portion of the action spectrum cannot be used to discriminate between these two possible types of photoreceptors. In the near-ultraviolet, where flavins have a strong peak matching the action spectrum peak, no carotenoid known to exist in the coleoptile absorbs heavily.

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MALEIC HYDRAZIDE-AUXIN INTERRACTIONS IN WATER UPTAKE OF POTATO DISCS[1, 2]

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Since the introduction of maleic hydrazide (MH) as a plant growth regulator by Schoene and Hoffman (17) there has been some speculation as to its role as an auxin antagonist. Many of the responses to MH of intact or isolated segments of plant tissue suggest some relationship to auxin processes. Thus, the cessation of growth, the prolonging of dormancy and the breaking of correlative inhibition have been related in some way to the auxin process and are also affected by MH. Cell elongation is to be considered similarly. Leopold and Klein (14) were among those who showed that MH had inhibitory effects on this phenomenon as measured in several common auxin bioassays. These workers, have indicated that MH is an antagonist of auxin action. Support for this aspect of MH action comes from the work of Kulescha (13) and Gautheret (8). However, the experimental approach used in these studies was one in which antagonistic effects could readily occur while masking other significant actions.

It is now generally accepted that a significant amount of the activity of auxins on the elongation of plant cells occurs through its influence on the cell wall. A loosening of cell wall constituents followed by an osmotic uptake of water is envisaged with a subsequent elongation of the cell (18). Increased aerobic metabolic activity has been shown to occur also during this period and a variety of cellular reactions have been followed in detail during these various stages under the influence of auxins.

In so far as the results of the present paper are concerned, the initial phases of auxin action are most significant. Other workers have shown that Avena coleoptiles incubated in the presence of auxin and isotonic or slightly hypertonic solutions of mannitol, can display auxin-induced elongation after the sections have been transferred to solutions where elongation may proceed and which contain no auxin themselves (4). These responses have been referred to as residual effects of auxin on cell walls. Such responses may also be readily observed by pretreating potato discs with auxins and following their subsequent uptake of water. This phenomenon has been utilized in the present studies in an effort to determine what effect MH has on this basic process under conditions of pretreatment and continuous incubation.

METHODS & MATERIALS

A technique similar to that used by Hackett and Thimann (11) was used throughout the course of this study. Katahdin potatoes were stored at 7° C until used. Discs 1 cm in diameter and 1 mm thick were cut using a cork borer and a hand microtome. After cutting, the discs were rinsed for 20 minutes in running tap water, given a brief distilled water rinse, and finally placed in petri dishes with enough distilled water to thoroughly wet the surface of the discs but not submerge them, and allowed to stand overnight at 25° C in a dark chamber. After 20 hours, the discs were removed in groups of ten, blotted by a standard procedure of placing them between double thicknesses of paper towel and applying a 100 g weight for 10 seconds, weighed on an electric balance to the nearest ten milligrams, and placed in the apparatus containing the test solution. This marked the beginning of the experimental period. The apparatus consisted of ordinary pyrex petri dishes containing two glass rods 5 mm in diameter, which supported circular pieces of coarse aluminum mesh heavily coated with paraffin. These served as supports for the potato discs. The test solution was added to the dishes until the liquid began to float the paraffin coated screens. This assured identical contact of the discs from dish to dish and thus assured uniform aeration. Each dish containing ten discs comprised an experimental replicate. Three replicates of each test solution were used in all experiments. Critical experiments which were repeated indicated that although the magnitude of the response of the discs varied slightly, the trend of the results was always the same. Data on the water uptake as effected by the various treatments was obtained by re-weighing the sections at 48 hour intervals during the 6-day experimental period. The weighing was performed in a manner similar to that used initially.

RESULTS

CONTINUOUS INCUBATION OF DISCS: Data illustrating the effect of naphthaleneacetic acid (NAA)

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TABLE I

EFFECT OF VARIOUS CONCENTRATIONS OF NAA ON STIMULATION OF WATER UPTAKE IN POTATO DISCS

TREATMENT NAA (ppm)	INITIAL FR WT (g)	WATER UPTAKE PERIOD (days) 2	4	6
		INCREASE IN FR WT (%)		
0.0*	1.11	6.3	10.8	12.6
0.01	1.11	5.4	11.7	13.5
0.1	1.12	4.5	17.0	21.4
1.0	1.10	6.4	32.7	49.1
10.0	1.11	8.1	37.8	55.9
50.0	1.11	0.9	10.8	14.4

* Distilled water control.

on the increase in fresh weight of potato tuber discs (table I) are very similar to those originally presented by Hackett and Thimann (12). A detectable stimulatory response is noted at a concentration of 0.1 ppm which increases in magnitude up to 10 ppm. It can generally be stated that a 0.01 g change in fresh weight is approximately equivalent to a one per cent change in this and subsequent tables. At 100 ppm toxic effects predominate (Data not illustrated). An important aspect of the data is that only small differences exist during the first 2 days of incubation. Later increases are much more pronounced.

The response of the discs to various concentrations of MH (table II) indicate that no pronounced inhibitory effects on the normal water uptake of the discs are noted until a concentration of approximately 20 to 50 ppm is reached. Thereafter, an increasing degree of inhibition ensues.

It is important to indicate here that all test solutions were aqueous and that the initial and final pH of the medium was not controlled on a routine basis. This was done for several reasons. First, it was found by buffer tests that the acidity of aqueous 100 ppm MH solutions (pH 4.9) was not a factor in the inhibition of water uptake in these tests (table III). Second, it was felt that difficulties in the interpretation of the data would arise in such tests, since the discs were exposed to the buffer salts for a comparatively long period of time and thus might be expected to be variously utilized in metabolic processes. Third, microbial activity would become troublesome.

In table IV data are presented on the interraction of the two chemicals. The MH concentration was varied while the NAA concentration was held at 1 ppm. By comparison with pertinent data in tables I and II, it is seen that 100 ppm MH almost completely negates the stimulatory action of the NAA. The lower concentrations of MH that were tested were ineffective in reducing the stimulatory action.

Data in table V describe the influence on water uptake of 100 ppm MH in combination with a series of NAA concentrations. It is shown that the stimulatory effects of NAA can be greatly or entirely suppressed. Again, pertinent data in tables I and II may be used for comparison.

TABLE II

EFFECT OF VARIOUS CONCENTRATIONS OF MH ON INHIBITION OF WATER UPTAKE IN POTATO DISCS

TREATMENT MH (ppm)	INITIAL FR WT (g)	WATER UPTAKE PERIOD (days) 2	4	6
		INCREASE IN FR WT (%)		
0.0*	1.13	6.2	13.3	14.2
0.01	1.11	5.4	12.6	15.3
0.1	1.12	6.3	13.4	15.2
1.0	1.11	5.4	12.6	14.4
10.0	1.11	7.2	13.5	15.3
20.0	1.12	6.3	10.7	11.6
50.0	1.10	5.5	9.1	10.0
100.0	1.13	5.3	8.0	8.8
500.0	1.13	2.7	6.2	3.5
1,000.0	1.10	−9.1	−37.2	

* Distilled water control.

PRETREATMENT OF DISCS: The foregoing data have been concerned with continuous exposure of the potato discs to various solutions of NAA and MH. The solutions supplied initially were never altered during the 6 day experimental period. While these data are important, they reveal very little about direct interraction phenomena between auxin-type growth

TABLE III

EFFECT OF BUFFERED & UNBUFFERED SOLUTIONS WITH & WITHOUT MH ON WATER UPTAKE IN POTATO DISCS

TREATMENT	INITIAL FR WT (g)	WATER UPTAKE PERIOD (days) 2	4	6
		INCREASE IN FR WT (%)		
Distilled water control at pH 6.9	1.12	7.1	11.6	13.4
100 ppm MH in water at pH 4.9	1.13	5.3	8.0	8.8
0.01 M pH 5 PO_4 buffer	1.11	6.3	11.7	14.4
100 ppm MH in pH 5 PO_4 buffer	1.12	5.4	8.0	8.9

TABLE IV

EFFECTIVENESS OF VARIOUS CONCENTRATIONS OF MH ON DEPRESSION OF STIMULATORY ACTION OF 1 ppm NAA*

TREATMENT MH—NAA (ppm)		INITIAL FR WT (g)	WATER UPTAKE PERIOD (days) 2	4	6
			INCREASE IN FR WT (%)		
0.0	0.0**	1.13	6.2	11.5	12.4
0.01	1.0	1.12	8.9	36.6	51.8
0.1	1.0	1.14	7.0	38.6	51.7
1.0	1.0	1.12	11.6	42.9	56.3
10.0	1.0	1.15	8.7	38.3	46.1
100.0	1.0	1.15	5.2	13.9	17.4

* As measured by water uptake in potato discs.
** Distilled water control.

stimulants and MH. A more direct approach to this problem is the pretreatment of the potato discs with the auxin followed by treatment with MH to study its influence on the residual auxin-induced water uptake. Conversely, pretreatments with MH, followed by treatments with NAA are of interest. Similar techniques have been used by workers in studies on auxin action (5).

A slight modification of the experimental procedure as described for continuous incubation of discs was required. The sections were weighed initially, incubated in the pretreatment medium for 48 hours, reweighed, transferred to the final solution, and weighed twice more at 48 hour intervals. Potato discs were thus pretreated with 1 ppm NAA and their subsequent response was observed after incubation in distilled water and several concentrations of MH (table VI). The total magnitude of the induced water uptake (24 %) is less than if the sections were continuously incubated in NAA (49 %). This increase is due entirely to the residual stimulatory effects of the NAA pretreatment. Similar phenomena have already been demonstrated (4). The present importance of these data lies in the inability of the MH final treatment to reduce the residual stimulatory action of the NAA pretreatment. Thus, the inhibitory influence of MH on auxin induced water uptake is negligible once some auxin action has proceeded.

The converse effect, the influence of an MH pretreatment on the subsequent response of potato discs to NAA, was also studied (table VII). When used as a pretreatment of potato discs 100 ppm MH causes a considerable reduction in the subsequent water uptake that can be induced by NAA. Thus, the MH pretreatment reduces the sensitivity of the potato discs to the action of subsequently applied NAA.

Experiments involving the inclusion of mannitol in the pretreatment medium have also been performed. Such techniques allow water uptake to be separated from the predisposing cellular alterations induced by auxins, which cannot be demonstrated with just aqueous solutions. An 0.2 M mannitol solution is sufficient to deter water uptake of the potato discs

TABLE V

EFFECTIVENESS OF VARIOUS CONCENTRATIONS OF NAA ON OVERCOMING INHIBITORY ACTION OF 100 ppm MH*

TREATMENT NAA—MH (ppm)		INITIAL FR WT (g)	WATER UPTAKE PERIOD (days) 2	4	6
			INCREASE IN FR WT (%)		
0.0	0.0**	1.16	4.3	12.9	13.8
0.01	100	1.15	4.3	10.4	12.2
0.1	100	1.16	5.2	11.2	12.1
1.0	100	1.15	5.2	13.9	17.4
10.0	100	1.16	2.6	21.6	25.0

* As measured by water uptake in potato discs.
** Distilled water control.

TABLE VI

EFFECTIVENESS OF FINAL TREATMENTS WITH SEVERAL CONCENTRATIONS OF MH IN REDUCING RESIDUAL STIMULATORY ACTION OF WATER UPTAKE*

MH** (ppm)	INITIAL FR WT (g)	WATER UPTAKE PERIOD (days) 2***	4	6
		INCREASE IN FR WT (%)		
0.0†	1.10	9.1	20.0	23.6
1.0	1.07	7.5	18.7	23.4
10.0	1.08	9.3	18.5	23.1
100.0	1.09	10.0	18.3	22.0

* Induced a pretreatment of potato discs with 1 ppm NAA.
** Presented 2nd through 6th day.
*** End of 1 ppm NAA presentation. Discs immediately transferred to MH solutions.
† Distilled water.

TABLE VII

EFFECTIVENESS OF 1 ppm NAA FINAL TREATMENTS IN OVERCOMING ACTION OF SEVERAL CONCENTRATIONS OF MH USED AS PRETREATMENTS

MH* (ppm)	INITIAL FR WT (g)	WATER UPTAKE PERIOD (days) 2**	4	6
		INCREASE IN FR WT (%)		
0.0***	1.15	6.1	13.9	22.6
1.0	1.16	6.9	15.5	25.0
10.0	1.14	6.1	13.2	21.1
100.0	1.10	4.5	9.1	11.8

* Presented for 2 days, from initial weighing to 2nd day.
** End of MH presentation. Discs immediately transferred to 1 ppm NAA solutions.
*** Distilled water.

TABLE VIII

EFFECT OF PRETREATMENTS OF NAA &/OR MH IN 0.2 M MANNITOL ON SUBSEQUENT POTATO DISC WATER UPTAKE IN VARIOUS AQUEOUS SOLUTIONS

TREATMENT (ppm)		INITIAL	WATER UPTAKE PERIOD (days)		
PRE*	FINAL	fr wt (g)	2**	4	6
0.2 M MANNITOL PLUS:			INCREASE IN FR WT (%)		
...***	H_2O	1.20	0.8	15.0	19.2
10 NAA	H_2O	1.20	1.7	27.5	39.2
10 NAA	100 MH	1.19	1.7	20.2	31.1
100 MH	H_2O	1.19	2.5	13.4	21.0
100 MH	10 NAA	1.20	2.5	10.8	20.0

* Presented for 2 days, from initial weighing to 2nd day.

** End of pretreatment presentation. Discs immediately transferred to final solutions indicated.

*** 0.2 M Mannitol only.

during the pretreatment period (table VIII). When NAA is included in the medium and is used as a pretreatment, apparently considerable auxin action proceeds in the presence of mannitol, even though water uptake is negligible in the first 48 hours. The auxin stimulation becomes evident after the discs have been placed in distilled water. The inclusion of MH in the final medium does not nullify the residual action of the NAA. These results resemble those given previously for NAA or MH pretreatments with no mannitol.

NATURE OF THE PROLONGED INHIBITION: Further investigations of the MH inhibitory phenomenon were attempted. The first phase involved the use of solutions of several inorganic salts immediately following pretreatment of the potato discs with MH to determine whether or not the inhibitory effects could be lessened by various ions active in physiological processes. The discs were pretreated with 100 ppm MH, immersed in the salt solutions for periods up to one hour, and then placed in 1 ppm NAA in an attempt to detect any lessening of the MH inhibitory influence. A 1 hour immersion in 1×10^{-3} M KCl was slightly effective. Other salts were practically without effect.

The second phase of the study concerning the nature of the prolonged MH induced inhibition in water uptake of potato discs involved the utilization of C^{14}-labelled MH [4]. The object of these studies was to determine whether or not the MH pretreated tissue retained the MH at the end of the 6 day experimental period. The basic test procedure was that used in the water uptake experiments with the exception that 200 lambda of a radioactive 1% solution of the diethanolamine salt of MH (specific activity 0.1 mC/mM) was added to 30 ml of aqueous medium containing a total of 200 ppm MH. Weighed discs were incubated on this medium for the treatment period and then removed, blotted, weighed, and immediately ground for 2 to 3 minutes in a mortar and pestle containing 2 ml of water. This material was washed quantitatively into a graduated centrifuge tube and the final liquid volume recorded. This was usually less than 10 ml. The material was centrifuged at a slow speed for 10 minutes to separate the ground residue from the supernatant liquid. A 1 ml aliquot of the supernatant was withdrawn, evaporated, and counted. The remaining supernatant was removed until a total volume of 2 ml of supernatant plus residue remained in the tube. The residue was then resuspended in this 2 ml volume by agitation and a 1 ml aliquot placed on a planchet for counting. The counts for many samples were quite low, often being only twice background. A thin window G-M tube was utilized throughout. Residue samples were necessarily counted at infinite thickness. All data have been corrected for background.

The discs were pretreated with radioactive MH for 2 days with a subsequent transfer to final treatments of either distilled water or 1 ppm NAA for 4 days. Discs were sampled at 48 hour intervals for 6 days after the experiment had begun. This experiment was repeated. Data of a typical experiment (table IX) show that under conditions of pretreatment with MH, radioactivity remained within the sections for the entire 6 day period. Further, a considerable amount of the activity remained in the residue fraction, which also showed a consistent increase in activity over the experimental period. Incomplete grinding could not account for this effect. Also, at-

TABLE IX

PERSISTENCE OF C^{14}-LABELLED MH IN POTATO DISCS EXPOSED TO A 2 DAY PRETREATMENT IN THE RADIOACTIVE SOLUTIONS OF MH*

FINAL MEDIUM	FRACTION	DAYS OF SAMPLING 2**	4	6
		(cpm/ml/g fr wt)***		
H_2O	Supernatant	397	267	217
	Residue	316	491	422
1 ppm NAA	Supernatant	334	205	211
	Residue	359	364	472

* After pretreatment, the sections were immediately placed in either H_2O or 1 ppm NAA for 4 days. Sample discs separated by grinding and centrifuging into a supernatant and residue fraction at each sampling day.

** End of pretreatment period.

*** cpm/ml/g fr wt. Sample fresh weight in grams and the final total sample volume in ml were expressed as g/ml. This value was the same for both the supernatant and residue fractions of a given sample. Final figure derived by dividing g/ml figure into observed count rate previously corrected for background.

[4] Kindly supplied by Naugatuck Chemical Corp., Bethany, Conn.

tempts to remove the activity from the residue with hot ethanol and hot 0.1 N HCl were only slightly effective. This suggests that the MH is held or incorporated into some component of the residue of the potato discs. Radioautographs prepared from chromatograms of the supernatant fractions which were developed in 8:1:1 isopropanol, ammonia, and water, showed the presence of MH at the end of the 6-day experimental period.

Discussion

Interractions in in vitro systems between auxins and MH have been the subject of several previous studies (7, 8, 13, 14). Leopold and Klein first suggested that MH be considered as an antiauxin. Their hypothesis was based on results obtained in several of the auxin bioassay tests. They have reported that the inhibitory action of MH on cell elongation may be completely overcome by auxins and inversely that the inhibitory effects of high auxin concentrations may be overcome by MH. The latter effect has been questioned by Foster (7) who could find no such action. Foster stated that MH was a disproportionate inhibitor in the presence of low concentrations of auxins and that the effect of MH in the presence of high auxin concentrations observed by Leopold must have been the result of some characteristic of the assay solutions. More recently, work by Pilet (16) and Kulescha (13) suggests that the effect of MH is not on the levels of auxins, but rather on their utilization, and therefore the action of MH on auxin systems is best described as that of an auxin antagonist. These effects are displayed as an antagonism of one chemical on the expression of the particular stimulatory or inhibitory reaction induced by the other. Experiments related to those discussed above have been carried out in the present work to obtain more information on the nature of the interractions of MH and auxins. The experiments were concerned with the effects of MH and NAA alone and in combination on water uptake in potato discs. Continuous incubation of the discs indicates that at certain levels of concentration NAA is stimulatory to water uptake and MH is inhibitory. This is in agreement with previously published data from related tests (1, 14). When the two compounds are mixed at appropriate levels, the interractions produced show clearly that the MH inhibition is the dominant influence of the system and that the MH inhibition reduces the stimulatory effects of the NAA on cell enlargement. Its inhibitory influences on water uptake in the absence of added NAA are small and could possibly arise through a secondary effect on a process unrelated to auxin utilization. The question of the role of cell division in these stimulative and inhibitory effects quite naturally arises. This process definitely occurs over the 6-day period (11) but its significance in regard to the total water uptake induced by treatments of 1 ppm NAA is probably of little importance. Thus, the inhibitory action of MH in this case does not seem to arise from its antimitotic effect.

It is of interest to consider briefly some of the basic aspects of auxin action as they relate to the possible effects MH might have on these processes. Evidence has been accumulating in recent years that the primary effect of auxins is on plant cell walls (5). It is generally agreed that the initial phases of auxin action are concerned with processes involving oxidative metabolism and that the result of these processes is a reduction of cell wall pressure (18). This initial phase of action can be separated in time from a later elongation phase. There are a variety of reactions occurring in the initial phase which auxins reportedly affect in a stimulatory manner (3, 9, 15, 19). What is more significant concerning these reactions is that they can occur prior to any determinable growth and once completed will allow elongation to proceed even in the absence of external auxin. Enlargement is accomplished by a subsequent uptake of water by a purely osmotic process governed by the previous loosening of the cell wall.

By the use of a 48-hour auxin pretreatment period, we have shown in this study that certain reactions have taken place in the potato discs during this period, which may later be displayed as an increase in water uptake in the absence of further auxin treatment. Likewise, when these reactions are allowed to proceed in potato discs for 48 hours, the induced water uptake which subsequently occurs cannot be greatly inhibited by treatments with MH. Only a slight diminution of the residual stimulatory effects induced by a pretreatment with NAA occur. It is suggested that this could be due to a secondary effect of MH which is independent of auxin activity. This effect of MH on auxin pretreated discs is in direct contrast to the much greater inhibitory activity of MH on water uptake when the two chemicals are mixed together and presented simultaneously. Therefore, MH is possibly antagonistic to some initial phase of the auxin-controlled processes leading to cellular expansion.

An important relationship to the above discussion exists in a consideration of the effect of pretreatments of MH on the subsequent response of the potato discs to treatments of NAA. Pretreatments of the discs in distilled water did not preclude a subsequent readily detectable stimulatory response to NAA. Thus, any processes occurring during these initial 48 hours in the absence of auxin, such as Hackett (12) has found to occur with the terminal oxidase systems, are not of themselves important factors to consider in the later response of the potato discs to NAA. However, it was shown that inclusion of MH in the pretreatment almost completely suppressed any stimulatory action exerted by a subsequent treatment of NAA in the absence of any external MH. This is evidence for the suppression by MH of a reaction involved in the initial phases of auxin action on cellular expansion, for the discs do not respond to auxin treatment.

The experiments utilizing mannitol in the pretreatment medium substantiate these data. Although negligible uptake of water occurred during the pretreatments, there were pronounced effects on subsequent water uptake by pretreating with either NAA or MH. MH, therefore, is not greatly affecting the processes that are directly related to osmotic water uptake but presumably has its effect on the reactions which occur prior to this water uptake.

An explanation of this residual inhibitory action of MH on cellular enlargement as affected by auxins would be of significance in the understanding of the nature of the inhibitory mechanism of MH and perhaps the nature of the auxin process itself since the MH molecule is unlike most auxin antagonists. Several exploratory experiments were carried out in the present work in an effort to gain further insight to these problems. An immediate question to be answered was whether it was the MH molecule itself within these potato discs that was causing the inhibition, or a derived complex molecule. Thus, experiments were carried out in an attempt to leach the MH pretreated discs with solutions of various inorganic salts. It was thought that if free or very loosely bound MH was causing the inhibition, these salts could possibly be exchanged for the MH in the cellular milieu and thus affect a reduction in the residual inhibitory action. However, if the MH were all tightly bound, no such reduction could result. Considering the possible stimulatory effects the various inorganic ions could have had on the potato discs, only slight relief in the residual inhibitory capacity was encountered, suggesting therefore, a possible binding of the MH to some cellular entity. The results of the experiments utilizing radioactive MH in the potato discs' incubation medium tend to confirm this supposition. Chromatographic results indicated that there was a certain amount of free MH in the discs for the duration of the experiment. Further, it was found that a considerable portion of the radioactivity within the discs was located on, and rather strongly affixed to, the solid material obtained on grinding the discs in distilled water. The data also indicate that the incorporation of radioactivity into the solid fraction increases with time.

Previous studies have indicated that much of the inhibitory action of MH is due to its effect on mitosis (6, 10). There are, however, several lines of evidence in addition to those presented in this study, which indicate that this supposition is not entirely correct. First, it has never been established that the anti-mitotic action of MH is a primary effect, thus allowing the assumption that its anti-mitotic action is a secondary effect due to the inhibition of a related process. This is entirely plausible when the variety of cellular reactions reported to be effected by MH are considered. Second, and perhaps most significant, is the fact that MH does not commonly show inhibitory activity in tissues other than those of higher plants (2). It seems possible then, that its influence could be exerted on some system which is unique to higher plant tissues. One readily apparent difference of cells of higher plant origin lies in the cellulosic nature of the cell walls where, presumably, the initial phases of auxin action occur. Certain processes occurring during this period have been shown to be affected by MH in the experiments previously discussed. The recent discovery of Towers (20) that MH forms a glucose glycoside upon incubation of wheat leaves in a medium containing MH and glucose is of importance also. The finding in the present study that MH tends to accumulate in the solid fraction of potato discs is suggestive of an affinity for certain constituents of the cell wall. The residual inhibitory influence of MH to any subsequent auxin-induced cell enlargement further supports an assumption that its activity is associated with some component related to cellular expansion. Towers has suggested that the glycoside found in wheat leaves constitutes a detoxification mechanism of the plant. In view of what has been presented here concerning the nature of the MH-auxin interaction, it is interesting to speculate how this glycoside, or some product related to it might be inhibitory to the process of auxin-induced cell elongation. The results obtained in this study suggest that MH does exert an inhibitory influence on auxin-induced cell enlargement, thus giving rise to the interactions heretofore demonstrated. Further evidence is required, however, to ascertain the significance of the apparent affinity of MH for cellular materials under the influence of added exogenous auxins.

Summary

The influence of MH and NAA on water uptake in potato discs has been studied. Both continuous incubation and pretreatment of the discs has been employed. These compounds were presented separately, successively, and in mixtures of varied concentration. With these experimental conditions, it was found that under conditions of continuous incubation MH can negate the stimulatory action of NAA on water uptake. The NAA apparently cannot overcome this inhibition. By pretreating the discs with NAA and then exposing them to MH, the inhibitory action of the MH does not appear to be effective. Conversely, pretreating the discs with MH renders them insensitive to any subsequent stimulatory action of the NAA. It is suggested that in this case the MH is acting on a process related to auxin-induced cellular expansion.

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OSMOTIC & SPECIFIC EFFECTS OF EXCESS SALTS ON BEANS[1]

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In analyzing the cause of growth depression of plants raised under saline conditions, a distinction can be made between effects that are specific and those that are non-specific, with respect to the different salt species present in the root environment (1). Non-specific effects are related to the total concentration of salts, regardless of species. They prevail among the factors that determine the partial molar free energy of water, from the gradients of which depend the magnitude of the driving force, as well as the direction pertinent to the translocation of water in both soils and plants. Known as osmotic effects, non-specific effects of salts are of a physico-chemical nature where they refer to the nutrient or soil-solution. They are barely distinguishable from physiological effects where they influence the state of water within the plants, as it may have bearing on cell processes. Both types of osmotic effects are associated to the extent that plants are capable of adjusting their internal osmotic pressure (OP) relative to changes brought about in the external OP (6, 8).

Specific effects are usually due to the relative concentrations of the various ion species with respect to one another, less frequently to the absolute level of any one element. Their influence is essentially of a physiological nature. Changes in the osmotic concentration within plant cells brought about by varying relative ion concentrations in the growth medium kept at a constant OP should also be recognized as specific effects. Osmotic effects are frequently confounded with specific effects because high total-salt levels normally occur in association with ionic conditions that are unbalanced with respect to plant nutrition. Other specific effects, like trace element toxicities, are usually unrelated to osmotic effects (27).

In many solution-culture studies purporting to separate the impact on plant growth due to osmotic effects from that due to specific effects, single salts (8, 9), mixtures of various salts (15), or organic compounds (10), have been used to control the OP of the solutions. Isosmotic solutions containing NaCl, Na_2SO_4, or $CaCl_2$, caused equal depressions in growth of kidney beans (9), and in water uptake by corn roots (10). Both water uptake and growth were linearly related to the concentrations of any of the three salts in solution. To the extent that isosmotic concentrations of different salts cause equal growth depressions, such as in the examples cited, osmotic effects are predominant. Less likely, though sometimes not impossible, is the alternative explanation that the specific effects of the different salts on the test plant are nearly similar. Any variation in plant response to isosmotic concentrations of different salts indicates the additional significance of specific effects. For instance, $MgCl_2$ and $MgSO_4$ depressed the growth of kidney beans significantly more than did NaCl, Na_2SO_4, or $CaCl_2$, all salts being compared in isosmotic concentrations. No proportionality existed between growth and Mg-salt concentration (9). With guayule as a test plant, neither was there a proportionality between growth and concentration of any one of the five salts mentioned, nor did the different salts when present in isosmotic concentrations cause the extent of growth depression to be the same. In fact, it appeared that $MgCl_2$ was most, and $CaCl_2$ least, toxic (24).

Attempts to distinguish between osmotic and specific effects on plant growth require the aid of a compound that, while lowering the partial molar free energy of water, at the same time itself is as inert as possible with respect to the metabolism of the plant species tested. The latter requirement has not always been sufficiently considered. Substances like polyvinylpyrrolidone (26) and polyvinyl alcohol (5) in concentrations equivalent to 1 atm OP have been observed to be toxic to dwarf red kidney beans (11). Other substances, like sucrose, are either actively taken up or, like mannitol, subject to quick microbiological degradation. The usefulness of the compounds mentioned for osmotic studies of any extended length of time is dubious. A search for an osmoticum of the desired qualities has resulted in the selection of Carbowax polyethylene glycol, of a molecular weight of about 20,000, marketed by the Union Carbide Chemicals Co. (11). This compound henceforth will be referred to as C2OM. Carbowax polyethylene glycols of lower molecular weights have been applied for various purposes, but no observations have been reported with regard to any physiological toxicity to plants (16). C2OM contains considerable amounts of aluminum and magnesium (11). These impurities would have to be removed prior to applying C2OM to most plants. Our paper will present details on the application of dialyzed C2OM to distinguish

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as well as possible between osmotic and specific effects of excess salts on dwarf red kidney beans grown to maturity in culture solutions.

Experimental Design & Methods

Two experiments were carried out with dwarf red kidney bean plants (*Phaseolus vulgare*, L.). The design of the first experiment accounted for ten treatments, repeated once, and represented by solutions different in their concentrations of NaCl, $CaCl_2$, and $MgCl_2$ (the latter two salts always present in normality proportions of 3:1), in the presence or absence of C2OM (table I). The salts were combined to give two values for the ion ratio $Na^+/\sqrt{Ca^{++} + Mg^{++}}$, viz., 3.1 and 22.5 calculated from activities derived from millimolar concentrations, and three values for the OP, viz., 1.3, 2.3, and 3.3 atm (cf. 12). The decimal value accounts for the presence of indispensable nutrients. The osmotic agent C2OM, after dialysis, was dissolved in concentrations of 6.2 % and 9.2 % by weight, equivalent to 0.7 and 1.2 atm OP in solutions already having an OP of 1.3 and 2.3 atm due to ions. The activity of the ions in solutions containing electrolytes only was calculated by applying the extended form of the Debye-Hueckel equation. The mean activity of NaCl in solutions containing C2OM was measured by means of the Na-selective glass electrode, a proven tool in saline (2) and medical (7) research. Corresponding values for the ionic strength were read from a graph relating the mean activity coefficients to the ionic strength on the basis of the equation mentioned. From these apparent ionic-strength values, the mean-activity coefficients and the mean activities of $CaCl_2$ and $MgCl_2$ in the C2OM-containing solutions have been calculated in the usual manner. The activity of the water followed from its relative vapor pressure as measured by a thermocouple psychrometer (18).

The second experiment was concerned with solutions characterized by two values for the ion ratio mentioned before, viz., 3.1 and 22.5, and two levels of OP, viz., 1.3 and 2.3 atm, (table I). Each of the four combinations was graded in three steps with respect to potassium chloride, so that the total K^+ concentration of the resulting 12 solutions amounted to 3.5, 8.8, or 14.1 meq per liter, equivalent to an added OP of less than 0.2, 0.4, or 0.6 atm, respectively.

Week-old seedlings were grown to maturity on the experimental solutions under Southern California greenhouse conditions during the periods extending from February 2 through March 22, and October 19 through December 2, for the experiments involving C2OM and K, respectively. The solutions were contained in 5-gallon jars, insulated by means of aluminum-backed fiber glass to reduce temperature variations. They were changed at least once a week to maintain a nearly constant solute concentration. Foam caused by aerating the C2OM-containing solutions was broken up by a half-inch wide strip of Dow Corning high-vacuum silicone grease, applied in a thin layer above the surface of the solution at the inside of the culture jar. Four plants, supported on a masonite lid with four openings, were grown in each jar. Upon terminating the growth, the fresh weight of various plant parts and the mineral content of the leaves were measured on samples representing the different treatments.

Of the ion species studied in acid-digested samples of plant material, Na and K were determined using the Beckman model 41 direct-reading flame photometer. Ca was measured by means of the Beckman DU flame photometer, after first removing possibly-interfering elements. The chloride data refer to the amount extracted with 0.05 N HNO_3 from dried and powdered plant material, and determined by electrotitration using an Ag-AgCl electrode in conjunction with a glass electrode and a pH meter.

The transpired water was measured by means of a water gauge. This consisted of a reading scale mounted behind the vertical arm of an L-shaped lucite tube that was fitted with its short arm in an outlet

Table I

Na^+, Ca^{++}, & Mg^{++} Content of Culture Solutions as Functions of Total Electrolyte Concentration & Ion Ratio* **

Total electrolyte conc in atm OP ***	Cation	Ion ratio * 3.1 Conc	3.1 Activity	22.5 Conc	22.5 Activity
1.3	Na	9.0	8.1	22.1	19.1
	Ca	15.0	9.8	1.9	1.0
	Mg	5.0	3.3	0.8	0.4
2.3	Na	13.0	11.5	41.0	34.3
	Ca	35.0	20.1	6.3	3.0
	Mg	11.7	6.7	2.1	1.0
3.3	Na	15.9	13.8	57.3	47.3
	Ca	56.1	30.8	14.7	6.3
	Mg	18.7	10.3	4.9	2.1
1.3 + 0.7†	Na	9.0	8.8	22.1	20.4
	Ca	15.0	13.0	1.9	1.3
	Mg	5.0	4.3	0.8	0.55
1.3 + 1.2††	Na	9.0	9.0	22.1	21.0
	Ca	15.0	14.5	1.9	1.5
	Mg	5.0	4.8	0.8	0.6

* $Na^+/\sqrt{Ca^{++} + Mg^{++}}$ calculated from activities, derived from millimolar concentrations. The Na, Ca, and Mg salts were added as the chlorides. One atm OP corresponds to a 0.024 N solution of NaCl, or a 0.032 N solution of $CaCl_2$.

** The concentrations are expressed in meq per liter. The pH of the solutions varied from 5.1 to 5.6.

*** Of these values, 0.3 atm is accounted for by K_2SO_4 (3 meq/l), NH_4NO_3 (2 meq/l), KH_2PO_4 (0.5 meq/l), H_3BO_3 (0.5 mg B/l), $MnCl_2 \cdot 4H_2O$ (0.5 mg Mn/l), and Fe-citrate (1 mg Fe/l).

† 0.7 atm accounts for Carbowax, 62 g/liter.

†† 1.2 atm accounts for Carbowax, 92 g/liter.

in the wall near the bottom of the culture jar. The rate of evaporation was measured from the decline of the water level in gauges attached to jars containing aerated solutions but no plants. Correcting the total uptake of water for the amount evaporated and for that incorporated in the plant (from the difference between fresh & dry weights), the transpiration was calculated per gram of dry weight. The OP of the leaf sap was deduced from freezing point depression measurements on composite samples collected after the cell walls had been destroyed by macerating the tissue using pure quartz sand.

Although no C2OM could be detected in the leaf sap, a white efflorescence appearing on the leaves of some C2OM-treated plants was identified by infrared analysis as polyethylene glycol (11). In these cases the occurrence of a shortening of the length of the molecular chain is likely, but molecular weight determinations fell beyond the scope of this study.

Results & Discussion

The results of the Carbowax experiment (table II) are averages of duplicate determinations and, in a number of cases, of their interpolations. Upon comparing the fresh weights of the various plant parts measured, we observed that adding inert C2OM to a base nutrient solution containing ions equivalent to 1.3 atm OP had caused growth to decrease sharply. Because changes in specific effects were excluded to the extent that no more than a negligible interaction took place between C2OM and ions present in a con-

Table II

Growth, Water Uptake, & Mineral Composition of Dwarf Red Kidney Beans*

OP atm	Ion ratio 3.1		22.5		Ion ratio 3.1		22.5	
	Ions	C2OM	Ions	C2OM	Ions	C2OM	Ions	C2OM
	Fr wt, *pods*, g				Fr wt, *leaves*, g			
1.3	79.4		63.3		81.6		61.8	
2.0**	38.3†	46.9	28.5†	37.4	54.0†	56.1	51.6†	53.0
2.3	25.7		20.6		44.0		44.7	
2.5***	20.0†	32.4	17.2†	29.8	39.0†	48.3	40.3†	45.3
3.3	7.5		5.9		22.8		17.2	
	Water transpired, ml/g dry wt				*% Dry matter of leaves*			
1.3	235		251		9.2		9.0	
2.0**	242†	221	267†	270	8.9†	11.1	8.7†	9.9
2.3	256		309		8.7		8.6	
2.5***	265†	255	319†	306	8.5†	10.4	8.4†	9.5
3.3	323		405		8.1		7.9	
	Na-content, leaves meq/100 g dry wt				*Ca-content, leaves* meq/100 g dry wt			
1.3	1.7		1.8		190		92	
2.0**	1.9†	1.8	2.5†	2.0	232†	206	155†	102
2.3	2.0		2.8		250		180	
2.5***	2.1†	1.9	3.0†	2.2	260†	220	196†	117
3.3	2.2		4.1		304		250	
	K-content, leaves meq/100 g dry wt				*Cl-content, leaves* meq/100 g dry wt			
1.3	100		194		123		109	
2.0**	86†	124	164†	238	188†	92	170†	82
2.3	81		150		211		198	
2.5***	79†	119	142†	189	228†	68	211†	59
3.3	73		108		279		268	
	OP of leaf sap, atm				(*OP leafsap*)-(*OP solution*), atm			
1.3	10.4		10.8		9.1		9.5	
2.0**	11.1†	8.4	11.4†	8.5	9.1†	6.4	9.4†	6.5
2.3	11.6		11.8		9.3		9.5	
2.5***	11.8†	9.0	12.2†	9.4	9.3†	6.5	9.7†	6.9
3.3	12.6		12.7		9.3		9.4	

* Raised in culture solutions containing an excess of ions (Ions), or Carbowax polyethylene glycol, molecular weight 20,000 (C2OM), at two ion ratios, and various osmotic pressures (OP).
** For C2OM-containing solutions: 1.3 atm due to ions, 0.7 atm due to C2OM.
*** For C2OM-containing solutions: 1.3 atm due to ions, 1.2 atm due to C2OM.
† By graphical interpolation, using data pertinent to solutions of 1.3, 2.3, and 3.3 atm OP.

centration equivalent to 1.3 atm OP (11), the decline in the growth rate must have been due primarily to osmotic effects. In turn, these may have influenced activities of a metabolic nature. Adding NaCl, $CaCl_2$, and $MgCl_2$ to a base nutrient solution of 1.3 atm OP in total amounts osmotically equivalent to that of C2OM, while maintaining a constant ion ratio $Na^+/\sqrt{Ca^{++} + Mg^{++}}$, caused the fresh weights of the plants to decrease even more. The difference in yield between kidney beans raised in solutions of 2.0 or 2.5 atm OP containing ions only, and those grown in isosmotic solutions containing C2OM should be credited to specific effects. These effects exerted a stronger influence at 2.5 atm OP of the solution where the excess of ions added was greater than at 2.0 atm OP. This trend allows for an extrapolation to the salt level of incipient significance of specific effects. In the present experiment, we can identify this value with an OP of the solution of 1.7 to 1.8 atm.

It appears that the OP of leaf sap of plants grown in solutions containing C2OM was from 20 to 30 % lower than that of comparable plants grown in isosmotic solutions of ions only. This is in agreement both with some of the ionic contents measured, and with the fact that the presence of C2OM in the leaves could not be established. With regard to the first point, the leaf contents of Na, and especially of Ca and Cl, are lower in the case of C2OM-treated plants as compared with plants exposed to ions only. The reverse holds for potassium, so much so, in fact, that the sum of the cation contents of the leaves of C2OM-treated plants is higher than the corresponding values for plants grown in isosmotic solution containing ions only. This may point to a higher leaf content of organic acids under the influence of C2OM in the nutrient solution. In view of the absence of C2OM in the leaves, the efflorescence of traces of Carbowax observed along some of the major leaf veins must be attributed to transport through the xylem by mass flow. The only plausible manner in which the polyethylene glycol macromolecules could have entered the xylem, is by permeating the meristematic tissue near the root tips prior to the formation of the endodermis.

One has to be aware of the characteristics typical for a lower OP of any one aqueous system, relative to that of any other system placed in the same surroundings. The partial molar free energy of the water is higher, i.e., the water binding forces exerted by the solute are weaker. As a result, the water molecules are at a greater freedom to participate in processes of uptake, growth, and transpiration (13, 19, 25). Under the experimental conditions, a lower OP difference between leaves and solution can be translated in terms of a correspondingly lower diffusion-pressure-deficit, or suction pressure, of the leaves. This implies that the force exerted by the leaves on the water in stems and roots, and the force keeping the water in the leaves from dissipating, viz., transpiring, also are weaker. The presence of leaf sap of a lower OP in the case of plants exposed to C2OM relative to plants raised in exclusively ionic solutions, therefore, might be indicative of the presence of water of greater availability but in a relatively critical supply. Evidence supporting this argument has been provided by data indicating that C2OM-treated plants were characterized by a higher dry-matter percentage, i.e. a lower moisture percentage relative to plants grown in isosmotic solutions, and in solutions of 1.3 atm OP containing ions only.

Of the same nature as the present work are studies dealing with osmotic recovery, involving Avena coleoptiles (3, 4, 22) and potato discs (21) exposed to hypertonic solutions of various compositions. In the latter study, potato discs lost water during exposure to solutions containing Carbowax, molecular weight 1500, and mannitol, and took up very little water from a mixed solution of KCl and $CaCl_2$. The solutions, all in the same molarity range, contained the auxin naphthalene-acetic acid (NAA). When subsequently exposed to a pure solution of NAA, the discs regained water. On comparison with control cultures, this osmotic recovery was complete in the case of salt solutions, whereas it was incomplete in the case of mannitol or polyethylene glycol. The explanation is that in the case of the salt solution the modification of the cell wall under the influence of NAA had continued, enabling the cells to recover osmotically when subsequently exposed to water containing NAA. On the other hand, impregnation of the cell wall with polyethylene glycol or mannitol had appeared to have interfered with the enzymatic activity enhancing cell-wall elasticity. In those cases, according to the Harvard group, return of the potato discs to water enabled them to regain but part of the water that ordinarily would have been taken up. In the present study, although kidney beans in the presence of C2OM in the culture solution had a lower internal water content, they were found to show more rather than less growth when compared to plants cultured isosmotically without C2OM. This would be in disagreement with any hypothesis regarding the interference of Carbowax with cell-wall growth of beans.

The higher leaf content of K, which element was supplied in equal concentrations in all solutions, must to some extent be related to the diminished competitive effect of those cations, especially Ca^{++}, of which a fraction was replaced by the polymer in C2OM-containing solutions. Note that in saline studies like the present one, the level of Ca is well beyond that at which this element exerts a synergistic effect on the rate of uptake of other cations (23). Rather, Ca and K behave antagonistically, as the data bear out.

Well-known studies (14, 17) have indicated the existence of an inverse relationship between the permeability of plant cells to solution and the valence of the major cation species taken up. It has been noted especially in the case of kidney beans exposed to mixed solutions of KCl and $CaCl_2$ in various ratios at constant Cl concentration (20) that the higher the K/Ca ratio of the plant, the more permeable the tissue to ions and water. In agreement, it has been shown

in the potassium experiment (table III) that the Na and Cl contents of the leaves increased as the K level was stepwise increased from 3.5 to 8.8 and 14.1 meq per liter at any one of the values for the OP and the ratio between Na^+ and $Ca^{++} + Mg^{++}$ ions of the culture solution. (For reasons mentioned, the Ca content meanwhile decreased somewhat.) As a result, the OP of the leaf sap also increased, and at a rate much greater than when the total salt level of the solution was raised by 25 to 30 meq per liter, or 1 atm OP added as NaCl, $CaCl_2$, and $MgCl_2$ at a constant ratio $Na^+/\sqrt{Ca^{++} + Mg^{++}}$ in terms of activities. This forms a good illustration of a change in the osmotic concentration of cell sap brought about by a specific effect. In turn, it causes an increase in the moisture percentage of the leaves. Phenomena of the same nature can be observed where the Na^+ concentration was increased relative to those of Ca^{++} and Mg^{++} at any one value for the OP or K^+ concentration of the solution.

As the K^+-concentration of the solution was stepped up, the total fresh weight of the plants decreased at the lower value for $Na^+/\sqrt{Ca^{++} + Mg^{++}}$ but it increased at the higher value for this ratio. These effects may both be explained on the basis of a suppressive tendency of K^+ with respect to Na^+ in relation to the absolute level of Na^+. This is because kidney beans are known to show maximum growth with neither insufficient[2] nor excessive amounts of Na^+ present in the nutrient solution (8). Admittedly, not only specific effects, but also osmotic effects may have been involved when the K^+ concentration was increased. For the very reasons that prompted this study, these effects cannot be separated in this instance. Nevertheless, the evidence presented validates the conclusion that growth is not contingent upon the absolute value of the OP of the plant system. By the same token, neither should the OP difference between plant and surroundings be considered as determining growth. This conclusion is in support of other pertinent statements (1).

[2] Unpublished data, U.S. Salinity Laboratory.

Summary

Osmotic and specific effects on dwarf red kidney beans have, to some extent, been separated by applying purified Carbowax (Union Carbide Chemical Co.) polyethylene glycol of a molecular weight of about 20,000 (C2OM). The plants were grown in isosmotic solutions of which the osmotic pressure (OP) was due to a base nutrient plus either an excess of NaCl, $CaCl_2$, and $MgCl_2$, or dialytically purified C2OM. Different ionic compositions were established by combining each of two values for the ion ratio $Na^+/\sqrt{Ca^{++} + Mg^{++}}$, calculated from activities, with each of a number of levels of OP. Results indicated that the yield of Carbowax-treated plants was greater than that of plants grown in isosmotic solutions containing ions only. The difference was attributed to specific effects of the salts mentioned. Extrapolation to the salt level of incipient significance of these effects was possible. The contents of Na, Ca, and Cl of the leaves and the OP of the leaf sap reflected the lower concentrations prevailing in C2OM-containing solutions relative to leaves of plants grown in isosmotic solutions containing ions only.

Table III

Growth, Water Uptake, & Mineral Composition of Dwarf Red Kidney Beans*

OP atm	Ion ratio 3.1			22.5			Ion ratio 3.1			22.5		
	a	b	c	a	b	c	a	b	c	a	b	c
	Total fr wt, g						*Water uptake/g* dry wt, ml					
1.3	693	662	637	636	670	738	248	216	252	252	224	224
2.3	532	485	452	534	544	562	252	236	204	224	200	208
	% Dry matter of leaves						OP *of leaf sap*, atm					
1.3	11.3	10.5	9.8	10.7	10.2	10.0	10.4	11.3	12.3	10.5	11.8	12.7
2.3	12.5	11.6	10.6	10.7	10.4	10.1	11.6	12.8	14.1	11.4	12.5	14.8
	K *Content of leaves*, meq/100 g dry wt						Na *Content of leaves*, meq/100 g dry wt					
1.3	132	162	180	158	195	235	2.2	2.5	2.8	2.2	2.4	2.7
2.3	120	142	152	145	166	192	2.4	2.8	3.0	2.5	2.7	2.8
	Ca *Content of leaves*, meq/100 g dry wt						Cl *Content of leaves*, meq/100 g dry wt					
1.3	173	193	168	98	88	62	134	195	234	110	139	168
2.3	260	280	238	180	175	135	187	244	276	191	206	227

* Raised in culture solutions containing an excess of ions at two ion ratios and two osmotic pressures (OP), both described in table I, and KCl to give total K^+-concentrations of 3.5 (a), 8.8 (b), and 14.1 (c) meq per liter.

The content of K was much higher in the leaves of C2OM-treated plants, probably due to diminished competition by Ca.

A separate experiment was carried out involving excess-salt-containing solutions similar to the ones used before except that they were graded with respect to potassium. Small increments of K caused significant increases in the OP of the leaf sap. From the results, in combination with those of the C2OM experiment, it can be pointed out that the growth of kidney beans, while strongly influenced by the OP of the nutrient solution, is not contingent upon the internal OP of the plant.

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SALT TOLERANCE & PROTOPLASMIC SALT HARDINESS OF VARIOUS WOODY & HERBACEOUS ORNAMENTAL PLANTS[1, 2]

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The interest in salt tolerance of plants, especially those of economic importance, is increasing as more saline lands are brought under cultivation, and as saline irrigation waters add to the salinity of cultivated soils. Research relating to the survival and growth of plants growing on soils containing excessive soluble salts has left many problems unsolved. The task of obtaining and selecting plants which may not only survive, but grow and produce satisfactorily on saline soils, is a varied and complex one. Hayward and Bernstein (2) recognized that salt tolerance may involve climatic, edaphic, genetic, physiological, and pathological factors and their interactions.

Salt hardiness of the protoplasm is perhaps the ultimate factor in salt tolerance of plants. Succulence, root distribution, and other factors may also contribute to salt tolerance, often by delaying lethal, internal salt concentrations (5). Repp and her associates (6) reported a rapid tissue test for relative salt hardiness. Stem sections were immersed in a graded series of sodium chloride solutions for 24 hours, after which their survival was tested by immersing them in hypertonic glucose solution. Cells which became plasmolyzed were considered alive, while cells which could not be plasmolyzed were considered dead. They found good correlation between the salt hardiness of the protoplasm and salt tolerance of a number of plants.

Van der Zweep (9) used plasmolytic studies in conjunction with tetrazolium for checking phytotoxicity of certain hydrocarbons on plants. He found that one technique substantiated the other. Since a better or easier technique is needed to interpret cell vitality after exposure to Repp's sodium chloride solution technique, tetrazolium was also employed in this study.

Of all plants studied thus far in relation to salt tolerance, ornamental plants, especially trees, have received relatively little attention; yet there is an increasing demand for practical as well as technical information concerning these plants.

In this study the protoplasmic salt hardiness of a number of ornamental species was measured by plasmolytic and tetrazolium tests. The salt tolerance of the same species was studied in solution culture or in artificially salinized salt plots and compared with the protoplasmic salt hardiness.

TERMINOLOGY. Terms relating to salt relations are used in this paper according to the following definitions suggested by Wright (Wright, J. L., personal communication).

SALT TOLERANCE. As used by Hayward and Bernstein (2), salt tolerance can be appraised in several ways: A, the ability of the plant to survive and grow on saline soil; B, the ability to produce a profitable yield, and C, the relative yield as compared to non-saline conditions.

SALT HARDINESS. This refers to the ability of cells or protoplasm to survive elevated internal salt concentrations. This term has been used by Levitt (4) although he did not define it. However, in speaking of frost or drought hardiness he uses the term "hardiness" to denote the ability of the plant to survive unfavorable internal environment. The above definition is analogous to Levitt's usage. In this study salt hardiness is measured by plasmolysis and tetrazolium tests, although other measures could be proposed. "Salt hardiness" would replace the term "protoplasmic salt resistance" as used by Repp et al (6). Salt hardiness would be a factor in salt tolerance, but salt tolerance might be due to other factors, such as succulence, which would delay the attainment of a critical internal salt concentration.

MATERIALS & METHODS

Two methods were used to study salt tolerance of different species. A variety of trees and shrubs was tested in artificially salinized plots in the field, while a number of herbaceous ornamentals were tested in solution culture.

FIELD EXPERIMENT WITH WOODY SPECIES. In May of 1958 an experiment was initiated to test the tolerance of some ornamental trees and shrubs grown on soil to various levels of salts.

Twenty plant species were used: ponderosa pine, *Pinus ponderosa* Laws.; blue spruce, *Picea pungens* Engelm.; eastern red cedar, *Juniperus virginiana* L.; douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco; black locust, *Robinia pseudoacacia* L.; honey locust, *Gleditsia triacanthos* L. var. inermis Willd.; Russian

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olive, *Elaeagnus angustifolia* L.; golden willow, *Salix alba* L. var. vitellina (L.) Stokes; black walnut, *Juglans nigra* L.; squaw bush, *Rhus trilobata* Nutt.; green ash, *Fraxinus pennsylvanica* Marsh var. lanceolata (Borkh.) Sarg.; tamarix, *Tamarix gallica* L.; little leaf linden, *Tilia cordata* Mill.; barberry, *Berberis thunbergii* DC.; winged euonymus, *Euonymus alatus* (Thunb.) Sieb.; Japanese honeysuckle, *Lonicera japonica* Thunb.; multiflora rose, *Rosa multiflora* Thunb.; buffalo berry *Shepherdia argentea* Nutt.; spirea, *Spiraea vanhouttei* (Briot) Zab.; arctic blue willow, *Salix purpurea* L. var. nana Hort.

All plants when obtained were seedlings approximately one year of age. The deciduous plants were straight stocks averaging 12 inches in length above ground. The evergreen plants (ponderosa pine, blue spruce, douglas fir, & eastern red cedar) averaged 6 inches in height. These plants were grown in artificially salinized field plots near Logan, Utah. The borders were made high enough so that approximately a four-inch depth of water could be easily held in each plot.

The plants were planted in pairs in a completely randomized design. Each pair of plants was replicated four times. The plants were irrigated with canal water approximately once each week until the treatments were begun on July 1. To avoid the shock which too abrupt a change in soil salinity might induce in the plant, the salinity was increased gradually during the first four salinizing irrigations.

Salt solutions consisted of equal parts of sodium and calcium chlorides dissolved in canal water. Five levels of added salt were applied 0—(control) 72, 108, 144, and 180 meq/liter. The treatments were continued for the remainder of the 1958 growing season. During the dormant season (March, 1959) all deciduous plants were pruned to a height of 18 inches.

At the beginning of the 1959 growing season, irrigations were resumed as before and continued once each week until Oct. 1, 1959, when the experiment was terminated and all plants were harvested.

Solution Culture Experiment With Herbaceous Plants. In the spring and early summer of 1959 a greenhouse experiment was initiated to determine the salt tolerances of eight species of herbaceous ornamental plants. The plants used in this study were as follows; pinks, double mixed, *Dianthus barbatus* L.; snapdragon, mixed, *Antirrhinum majus* L. horticultural var. Super Majestic; petunia, *Petunia hybrida* Vilm. horticultural var. nana compacta; Celosia, *Celosia argentea* L. var. Cristata Kuntze, horticultural var. Toreadore; moss rose, single mixed colors, *Portulaca grandiflora* Hook.; alyssum, Little Gem, *Alyssum saxatile* L.; zinnia, giant mixed dahlia flowered, *Zinnia elegans* Jacq.; and kochia, *Kochia scoparia* Schrad. var. culta Farwell.

The seeds were germinated in flats containing a horticultural grade of vermiculite. When the young plants were all approximately three weeks of age, they were transplanted to 3.5 liter glass jars containing aerated Hoagland's solution no. 2 (3) at half strength. The pH of the solution culture was adjusted to 6.0 by using 0.1 N sulfuric acid, and iron chelate (Na Fe diethylenetriamine pentaacetate) was added every other day. Once each week all solutions were changed.

The following treatments were begun, and were continued for 3 months: Control, Hoagland's solution no. 2 diluted to half strength with tap water.

60 meq, Hoagland's solution no. 2 diluted to half strength with tap water, plus 30 meq calcium chloride and 30 meq sodium chloride per liter.

120 meq, as above, plus 60 meq calcium chloride and 60 meq sodium chloride per liter.

180 meq, as above, plus 90 meq calcium chloride and 90 meq sodium chloride per liter.

All treatments were replicated four times in a completely randomized design, making a total of 128 solution cultures. Four plants were grown in each of the jars containing the solution cultures, making a total of 16 plants in each treatment.

Protoplasmic Salt Hardiness. Fresh stem samples were taken from the control plants for protoplasmic salt hardiness studies. For these studies a technique described by Repp et al (6) was used with some modifications. Free-hand tangential sections containing the epidermis and several subjacent cells of the lower part of the stem were placed for 24 hours in solutions of the following concentrations of sodium chloride: 0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50 molar, in 0.1 strength Hoagland solution. These sections were then placed in a hypertonic sucrose (1.5–2 molal) solution for 4 hours. Viability of the cells was then determined by examining them under the microscope. If 50 % or more of the cells became plasmolyzed, the tissue was considered to be alive. Those which did not plasmolyze were considered to be dead. The highest molar salt concentration in which the tissues were considered to be alive was recorded as the critical salt concentration.

Many problems were associated with the performance and interpretation of the plasmolytic method so another method was investigated. These problems include small cell size, inability to cut thin sections and abnormal plasmolysis.

Many workers (7, 8) have used 2, 3, 5-triphenyl tetrazolium chloride (TTC) as a vital stain; consequently this dye was utilized in connection with this work. A 1 % solution was found to be unsatisfactory. Van der Zweep (9) pointed out that tetrazolium salts are quaternary ammonium compounds and that these compounds are toxic to living cells above 2.5 %, so attempts were then made to use weaker solutions. The 0.1 % solution proved best for tissues from woody species, and a 0.05 % solution proved best for tissues from herbaceous plants. The most consistent results were obtained if 0.1 strength Hoagland's solution no. 2 was used as a base solution instead of distilled water.

In order to get tissue samples which would produce consistent results, samples were taken from various parts of the stem, buds, and leaves. Stem sections split longitudinally through the tip proved most satisfactory. All sections were taken from healthy control plants. The samples were placed in sodium chloride solutions made up with 0.1 strength Hoagland's solution: A, 0; B, 0.25 molar; C, 0.5 M; D, 0.75 M; E, 1.00 M; F, 1.25 M; G, 1.50 M, and H, 0 + tetrazolium solution 0.1 or 0.05 %.

After 24 hours the salt solutions were decanted and the stem sections rinsed once with tap water. Then enough 0.1 or 0.05 % TTC solution was added to each vial to cover the sections. At the end of an additional 24 hours they were examined for red coloration of the tissue. If the sections appeared red or pink, depending on the plant species and the amount of coloration found in comparison with those in the control solution, they were considered to be alive. We recorded the highest molar concentration in which the sections were considered as being alive. All samples were checked against those in vial no. 8 containing only the TTC solution. If no red coloration was found, all samples were discarded and a new group of tissue samples was taken. For development of good color in these stem sections, 24 hr was necessary, in contrast to the much shorter periods of time required for color development in seeds or meristematic tissues.

Results & Discussion

Field Experiment With Woody Species. When the salt irrigations were first applied in full strength on July 21, 1958, all of the plants appeared to be in excellent condition and were making good growth. By September 15, some of the leaves on some of the plants in the plots receiving the salt applications were becoming chlorotic. In the plots receiving the greatest amount, 180 meq/1 of salt, all of the deciduous trees except the squaw bush, green ash, black locust, honey locust, Russian olive, golden willow, tamarix, and honeysuckle had lost their leaves. On all the evergreen species in this plot the needles had turned red-brown and had become brittle. All plants on the control plot had a normal, healthy green appearance on Sept. 15, 1958.

In April 1959, all plants which had retained their leaves or that had green needles in the preceding fall were still alive. As the 1959 growing season progressed, more and more plants showed signs of salt sensitivity. The leaves turned yellow and/or tipburn occurred. By July 1, all plants which appeared to be salt sensitive had lost their leaves, shown signs of marginal leaf necrosis, or their leaves had turned brown and remained hanging on the plants. There was no visual evidence of any additional damage after July 10 in any of the plots. Plants which had survived to July 10 survived for the remainder of the

Table I

Salt Tolerance & Salt Hardiness of Various Trees & Shrubs

Species		Field survival	Tissue survival	
			Plasmolysis	Tetrazolium
Blue spruce	*Picea pungens*	0*	250**	250**
Black walnut	*Juglans nigra*	0	0	500
Douglas fir	*Pseudotsuga menziesii*	0	0	750
Linden	*Tilia cordata*	0	0	750
Barberry	*Berberis thunbergii*	0	0	750
Euonymus	*Euonymus alatus*	0	0	750
Rose	*Rosa multiflora*	0	0	750
Spirea	*Spiraea vanhouttei*	0	0	750
Blue willow	*Salix purpurea* var. nana	0	0	500
Ponderosa	*Pinus ponderosa*	108	500	500
Red cedar	*Juniperus virginiana*	108	250	750
Green ash	*Fraxinus pennsylvanica* var. lanceolata	108	500	1,000
Honeysuckle	*Lonicera japonica*	108	750	750
Golden willow	*Salix alba* var. vitellina	144	750	1,000
Buffalo berry	*Shepherdia argentea*	180	1,000	750
Black locust	*Robinia pseudoacacia*	180	1,000	1,500
Honey locust	*Gleditsia triacanthos*	180	1,250	1,250
Russian olive	*Elaeagnus angustifolia*	180	1,500	1,500
Squaw bush	*Rhus trilobata*	180	1,000	1,500
Tamarix	*Tamarix gallica*	180	1,000	1,500

* Concentration in meq/liter of NaCl and $CaCl_2$ added in equal parts to irrigation water at which plants survived for two growing seasons.

** Concentration in meq/liter of 0.1 strength nutrient solution in which tissues survived for 24 hr.

growing season; sensitive plants had been killed by this time.

The highest salt concentration at which each species survived is given in table I.

Solution Culture Experiment With Herbaceous Species. When treatments were started, all plants of the same species were of the same size and all had made luxuriant growth. The first observable effect of the salt treatment was incipient wilting of the lower leaves. The severity of symptoms increased with time and finally resulted in the death of many plants.

The plants of all species in the control solution remained in good condition throughout the experiment, which extended through the flowering stage. Petunia and portulaca were not visibly affected by salt at the highest level studied. Some of the kochia plants survived the highest salt level, but approximately one-fourth had died in level 3 (120 meq/1) and one-half had died in level 4 (180 meq/1).

Table II shows the highest salt concentration at which each species survived.

Tests for Protoplasmic & Salt Hardiness. The survival of cells and tissues after 24 hr in salt solutions, as measured by the plasmolysis and tetrazolium tests is given in tables I and II. Both tests compare quite favorably with each other and with salt tolerance. Several factors could account for discrepancies between the two tests: A. It was difficult to obtain and interpret results of the plasmolysis test with some species because of small cell size or some intrinsic factor related to preparation of the tissue for this test. Cutting of tissue may have resulted in plasmolysis of some cells, especially of those adjacent to the cut. B. As pointed out by Repp (6), the vacuole in a damaged cell may be easily mistaken for the normal, living plasmolyzed cell contents. In cells where death is due to salt the vacuole may appear normal but the cytoplasm will appear more granular; sometimes the cytoplasm is in clumps bordering the vacuole. In some instances it was difficult to get any plasmolysis other than that caused by direct injury of the cells in cutting. This problem was partly solved by using concentrated sugar solutions (2 molal).

In many species the tetrazolium test indicated tissue hardiness in somewhat higher salt concentrations than did the plasmolysis test. This is perhaps not too surprising since the tetrazolium test is a measure of enzyme activity, and many enzymes are studied after the cell has been disrupted. Enzymes may, and often do, remain active under conditions under which the cell is no longer viable. An intact plasma membrane is necessary if the cell is to plasmolyze and it is likely that a number of complex reactions are necessary to maintain the membrane, and that it is therefore relatively sensitive to disturbance. Currier (1) pointed out that plasmolytic studies involve many changes in cell structure.

A correlation was found between the tests for protoplasmic salt hardiness and salt tolerance in field or solution cultures. Plants which were able to survive in the highest concentration of salt in field plots also had tissue which was resistant to the greatest concentration of salts as determined by the two tissue tests. Comparison of the TCC test with the survival of plants in the field indicated that plant tissue from plants that were somewhat more tolerant to higher salt treatments in the field remained viable after exposure for 24 hr to sodium chloride solutions greater than 0.75 molar. Plants with the greatest resistance to sodium chloride (i.e., remained viable in 1.5 molar NaCl solution) were without exception the ones able to survive in the field plot receiving the highest level of salt (180 meq/1). Also it was noted that plant tissues which produced a red formazan from TTC after exposure to salt up to 0.75 molar did not in some cases exhibit salt tolerance in the field tests. This seems to indicate that the TTC test used in conjunction with the Repp technique is reliable for the salt tolerance appraisal of plants only if the red forma-

Table II

Salt Tolerance & Salt Hardiness of Various Herbaceous Ornamental Species

Species		Plant survival	Tissue survival	
			Plasmolysis	Tetrazolium
Celosia	*Celosia argentea*	0*	500**	500**
Alyssum	*Alyssum saxatile*	0	250	250
Pinks	*Dianthus barbatus*	60	250	250
Snapdragon	*Antirrhinum majus*	60	250	250
Zinnia	*Zinnia elegans*	60	250	250
Kochia	*Kochia scoparia*	180	500	1,000
Petunia	*Petunia hybrida*	180	1,000	1,000
Portulaca	*Portulaca grandiflora*	180	750	1,000

* Concentration in meq/liter of equal parts NaCl and $CaCl_2$ added to the nutrient solution at which plants survived and grew.

** Concentration in meq of NaCl per liter of 0.1 strength nutrient solution in which tissues survived for 24 hr.

zan is formed in the tissue after it has been subjected to salt concentration in excess of 0.75 molar for 24 hr.

Both tissue tests suggest survival at much higher salt concentrations than the field tests. The tissues tests were limited to 24 hr, while the field tests required from 2 months to 2 years. If the tissues tests had been conducted for 2 days or a week instead of 1 day it is probable that the salt concentration critical for survival would have been much lower. Such extended tests with excised stem sections would, however, be complicated by the moribund changes associated with excision. Even the relatively brief 24-hr studies reported here are undoubtedly complicated by factors such as wound injury, osmotic shock, and plasmolytic damage. These may account for the lack of closer correlation between the various tests.

These tests were conducted on sections taken from the control plants grown without added salt, and therefore represent normal healthy tissue. The influence of growth in saline soils on salt resistance of the protoplasm was not studied. These studies therefore give information on the innate or genetical protoplasmic salt hardiness of different species; they give no data on salt hardening.

Summary

The salt tolerance of 28 species of woody and herbaceous plants was studied. The trees and shrubs were grown in salinized field plots, while the herbaceous plants were grown in salinized solution cultures.

Tissue samples of the same species were measured for salt hardiness by the following techniques: A. Plasmolytic method: 1, immersion of tissue sections in a graded series of sodium chloride solutions for 24 hr, followed by 2, immersion of sections in hypertonic sucrose solution for 4 hr, and 3, microscope examination for plasmolysis. The highest salt concentration at which the majority of the cells still became plasmolyzed was used as a measure of salt hardiness. B. Tetrazolium method: 1, step 1 above, 2, immersion of sections in a 0.1 % or 0.05 % solution of 2,3,5-triphenyltetrazoliumchloride for 24 hr, and 3, examination of sections for pink or red formazan color. The highest salt concentration at which the red color developed was used as a measure of salt hardiness.

A good correlation was found between salt tolerance in field or solution cultures and both the plasmolytic and tetrazolium tests for salt hardiness.

Salt tolerant species were golden willow, *Salix alba* var. vitellina; buffalo berry, *Shepherdia argentea;* black locust, *Robinia pseudoacacia;* honey locust, *Gleditsia triacanthos* var. inermis; Russian olive, *Elaeagnus angustifolia;* squaw bush, *Rhus trilobata;* tamarix, *Tamarix gallica;* kochia, *Kochia scoparia* var. culta; petunia, *Petunia hybrida*; portulaca, *Portulaca grandiflora.*

The following species were not salt tolerant: blue spruce, *Picea pungens,* black walnut, *Juglans nigra;* linden, *Tilia cordata;* barberry, *Berberis thunbergii;* euonymus, *Euonymus alatus;* rose, *Rosa multiflora;* spirea, *Spiraea vanhouttei;* blue willow, *Salix purpurea* var. nana; douglas fir, *Pseudotsuga menziesii;* celosia, *Celosia argentia* var. cristata; alyssum, *Alyssum saxatile;* pinks, *Dianthus barbatus;* snapdragon, *Antirrhinum majus;* zinnia, *Zinnia elegans.*

The following species were somewhat salt tolerant: red cedar, *Juniperus virginiana;* ponderosa, *Pinus ponderosa*; green ash, *Fraxinus pennsylvanica* var. lanceolata; honeysuckle, *Lonicera japonica.*

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SUSPENSION CULTURES OF HIGHER PLANT CELLS IN SYNTHETIC MEDIA[1,2]

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The cultivation of cells from higher plants is a technical advance in plant tissue culture research which opens new avenues of investigation into cellular and physiological problems. Successful culture of higher plant cells as suspensions in liquid media of complex constitution has been reported by several research groups.

Isolated cells or cell suspensions produced in culture have been used in a variety of problems, including the study of cell division [Muir et al, (9, 10); deRopp (5); Torrey (18); Steward et al, (16); Braun, (4); Jones et al, (7)], susceptibility of tissues to virus infection [Hildebrandt, (6); Bergmann, (2)], cellular differentiation [Steward et al, (15); Reinert, (12)], the production of cell metabolites [Tulecke & Nickell, (21)]. Still many problems remain untouched. The production of cell suspensions has been achieved to date only with complex nutrients such as coconut milk, yeast extract, or other complex media, which limit the usefulness of the technique, especially where analysis of chemical changes is desired. Our purpose is to report relatively simple techniques devised to allow the cultivation of large numbers of isolated viable cells in suspension under defined nutrient and cultural conditions. We give an account of the course of development of liquid cultures with respect to increases in fresh weight and numbers of cells in suspension.

MATERIALS & METHODS

Several techniques have been reported for cultivating plant cell suspensions in complex media. Steward et al (14), and Steward and Shantz (17) described the cultivation of carrot tissue in a specially-designed culture tube slowly rotated end-over-end around a horizontal axis. For large volumes of tissue, they designed a 1-liter flask with projecting nipples which was similarly rotated continuously. Explants of carrot root cultivated in a coconut milk medium underwent rapid proliferation with sloughing of cells into the medium. Repeated subculture of cell suspensions in liquid samples pipetted into fresh medium allowed continuous cultivation of these cells in suspension [Steward et al (16)]. Nickell (11) cultivated in cell suspension callus tissue from pole bean hypocotyl. Agar-grown callus tissue pieces were transferred into 300 ml Erlenmeyer flasks containing White's medium supplemented with coconut milk and 2,4-dichlorophenoxyacetic acid (2,4–D) and were agitated continuously by various devices. Subcultures were established from pipetted aliquots taken at monthly intervals. Tulecke and Nickell (21) described methods for producing large amounts of cell suspensions using 20-liter carboys containing 9 liters of complex medium. Cell suspension and aeration were achieved by continuous bubbling of filtered air. Inoculation of the carboys was made with large volumes of liquid cultures developed by successive passage in 300-ml Erlenmeyer flasks and thence to 900-ml volumes in Fernbach flasks. Rapid increase in rate of formation of cells in suspension was attributed to the serial cultivation procedure. Muir et al (10) studied growth in cell suspension of a number of callus tissues of crown-gall origin, cultivating tissues in various volumes of medium in 6-oz prescription bottles agitated on a variety of rotating and shaking devices. The most rapid growth in a complex medium was achieved with a reciprocal shaker operating at 60 cycles per minute. Melchers and Bergmann (8) described an elaborate culture system for the cultivation of tissues of Antirrhinum in which the liquid in a flask was agitated continuously with a magnetic stirrer and a continuous inflow of filtered air. Tissue growth in a complex medium with coconut milk and 2,4-D was in the form of small aggregates but with few cells.

In our own earlier studies [Torrey & Shigemura, (20)] pea root callus tissue was grown in liquid medium containing yeast extract and 2,4-D in 125-ml Erlenmeyer flasks continuously agitated at about 120 rpm on a horizontal-type rotary shaker. In those experiments, it was observed that separation of cells was not primarily dependent upon agitation of the cultures, which was, however, essential for aeration and thus for tissue growth, but rather tissue friability was related to the constitution of the nutrient medium,

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in which the interaction of yeast extract and 2,4-D played a controlling role.

In the experiments described here, attention was focused on the role of the nutrient medium in favoring cell suspension growth and the mechanical manipulation of the liquid cultures was simplified as much as possible.

CULTURAL CONDITIONS. The following procedure was used in the present studies. Twenty-five milliliters of nutrient medium in a 125-ml Erlenmeyer flask were inoculated aseptically with a callus tissue piece of about 100 mg fresh weight taken from an agar-grown culture. After inoculation, the mouth of the flask was enclosed with aluminum foil and the flask was placed on a rotary horizontal shaker and agitated continuously in a constant temperature room at 25° C. The data are for experiments conducted in the dark, but other experiments suggest that continuous diffuse fluorescent light does not significantly affect the growth rate or the separation of cells. The rate of agitation tested in various experiments was from 80 to 160 rpm. At the higher rotation rates, growth was faster but cells were lost due to deposition high on the side of the flask wall. Best results were obtained at rotation rates of 80 to 120 rpm. The commercial gyrotory rotary action flask shaker (New Brunswick Scientific Co.) was quite satisfactory.

PLANT TISSUES & NUTRIENT MEDIA. During these experiments, a number of plant tissues were studied. In each case, callus tissues which had been previously well established in agar tube culture on complex media were used for inocula. A clone of carrot tissue (*Daucus carota* L.) growing on White's medium + 5 % coconut milk + 5×10^{-8} g/ml 2,4-D previously studied by Reinert (12) was used; a clone of root callus tissue isolated from excised root cultures of *Convolvulus arvensis* L. was maintained in an agar medium (M6) devised earlier (20) for pea root callus containing 1 g/l yeast extract and 10^{-6} M 2,4-D. Also tested was a clone of callus tissue derived from the hypocotyl of seedlings of *Haplopappus gracilis* (Nutt.) Gray maintained on White's medium +5 % coconut milk + 5×10^{-8} g/ml 2,4-D (13). All three of these tissues were cultured successfully as cell suspensions in the synthetic medium described below. Detailed data are presented for Daucus and Convolvulus. Attempts to culture tissues of pea root callus on the same synthetic medium were unsuccessful, although success has been achieved with the synthetic medium devised by Shigemura [Torrey, (19)].

The synthetic medium used by Reinert (12) was the basic medium used in the cell suspension cultures to be described. Although a complete analysis of the role of each component in this medium or its essentiality for continuous culture of the various tissues has not been conducted, there is clear evidence that the medium can be effectively used in propagating cell suspension cultures and that this medium gives rise to cell separation as great as any of the complex media previously reported. Some evidence concerning those components of the medium which are particularly critical in influencing cell separation is presented below.

The complete synthetic medium (S3) contained the following components:

I. White's medium in mg per liter: 200 $Ca(NO_3)_2$; 200 Na_2SO_4; 80 KNO_3; 65 KCl; 16.5 NaH_2PO_4, 360 $MgSO_4$, 4.5 $MnSO_4$; 1.5 $ZnSO_4$; 1.5 H_3BO_3; 0.75 KI; 2.5 $Fe_2(SO_4)_3$; 3.0 glycine; 0.1 thiamine, 0.1 pyridoxine; 0.5 nicotinic acid. These weights should be adjusted for water of hydration or hydrochlorides. To these are added 20 g sucrose and glass distilled water to make the final solution.

II. Additional vitamins in mg/l: 100 inositol; 10 choline; 0.1 riboflavin; 0.1 ascorbic acid; 0.1 calcium pantothenate; 0.01 biotin.

III. Purine in mg/l: 2.5 hypoxanthine.

IV. Amino acids and amides (stock solution adjusted to pH 5.5) in mg/l: 6.0 aspartic acid; 7.8 L-arginine HCl; 1.5 L-cystine; 14.0 L-glutamic acid; 10 glycine; 2.6 L-histidine; 10.4 D,L-isoleucine; 15.6 L-leucine; 15.6 L-lysine; 13.0 D,L-methionine; 2.5 L-phenylalanine; 5.0 L-proline; 6.5 L-threonine; 4.0 L-tryptophan; 40 L-tyrosine; 13.0 D,L-valine; 50 L-glutamine, 20 L-asparagine.

V. Auxin in mg/l: 0.05 2,4-dichlorophenoxyacetic acid.

The components of White's medium were autoclaved together; the remaining components, in separate stock solutions, were cold-sterilized through membrane filters (Millipore) and added to make up the final solutions. The final pH of the medium was pH 6.0. Because of their low solubilities, hypoxanthine and L-tyrosine stock solutions were made up ten times more dilute than other stocks and added to the final solution just before making up to final volume. Variation in the constitution of the S3 medium consisted largely in the omission of one or more components of the medium.

MEASUREMENT OF TISSUE GROWTH & TISSUE DISSOCIATION. In no attempts thus far made, either in published reports or in our own studies, has it been possible to cultivate higher plant cells in suspensions in which all cells are single and free-floating. This is an ideal yet to be reached. In all studies, single cells, cell pairs, groups of from three to ten cells, and larger clumps are found in growing cultures, even when one begins a culture with a filtered single-cell suspension. Such single cells divide and rapidly form small clumps [cf. Steward et al, (15, 16)] and only later do cells slough off from the larger pieces to produce a cell suspension. This characteristic type of growth raises certain difficulties in analyzing the

course of development of cell suspension cultures in liquid media. Thus it has been necessary to devise methods for separating and studying separately the cellular and the cell aggregate components of the cultures. The following methods were developed in the present work and were arbitrarily applied to all cultures. They have the merit of allowing comparisons between different treatments; the data reliably reflect in a relative way changes in growth under different nutrient conditions.

Since interest centered on the separation of cells (tissue dissociation) accompanying tissue growth, an estimate was made of the proportion of the total cell mass which was freely suspended in the liquid medium. At the time of sampling, the entire contents of a flask culture were poured into a glass cloth filter supported in a funnel. The filter was of fine mesh which allowed cells up to about 100 μ diameter to pass. The solid tissue pieces which did not pass through the mesh were collected and fresh weight and dry weight determinations were made for the particulate fraction of the culture. The filtrate contained single cells, and pairs or groups of cells up to approximately 100 μ in diameter. A 1-ml aliquot of the stirred filtrate was removed and an estimate of cell number was made, using the Sedgewick-Rafter counting cell with microscopic magnification at about 100 ×. The remaining cell suspension of measured volume was centrifuged in conical centrifuge tubes at about 500 *g* until a packed cell volume could be determined. For cytological examination, the clear supernatant was removed. The cells were fixed in 45% acetic acid for about one minute and then squashes made using lactic-acetic-orcein stain [Beerman (1)]. From such squashes, the per cent of cells in mitosis could be determined.

In analyzing the effects of the medium on tissue separation it was interesting to calculate for each nutrient condition the per cent of the total fresh weight of each culture which was present as cell suspension. These figures were calculated from the measured fresh weight of the particulate fraction and the fresh weight of suspended cells calculated from data on cells per ml based on the total supernatant cell population. An estimate of average fresh weight per cell was made as follows:

Average number of cells per ml (C) × Average dry wt per cell in mg (D) × Volume of supernatant in ml (V) × [Mean fr wt of pieces (A)/mean dry wt of pieces (B)] = calculated fresh weight of cells in suspension in mg (E) and $\frac{E}{A + E} \times 100 =$ % of total culture fresh wt in suspension.

The estimation of the average dry weight per cell in suspension (value D above) was made for each tissue in the following way. A small circle of fine filter paper was weighed accurately and then a measured volume of cell suspension (from a sample whose average cell content had been determined by the cell counting procedure described above) was passed through the filter paper which trapped all the cells. The circle of paper (with the cells adhering) was dried to constant weight in a drying oven at 80° C and the paper was reweighed. The difference in weight was cell dry weight; from this value and the estimate of cell number per ml a direct calculation of average dry weight per cell could be made. For several such determinations the mean value for carrot was 2×10^{-5} mg per cell and for Convolvulus 1.5×10^{-4} mg per cell. Variation in average cell weight in different media was remarkably small for either tissue but the tenfold difference in average cell weight between the two tissues was consistent. No attempt to determine cell number in the particulate fraction of the cultures was made, so no comparison of tissue proliferation on a total cell basis was possible in these experiments.

In several experiments, propagation of liquid cultures was made using inocula composed of cell suspensions themselves, obtained after filtering once through glass cloth or repeatedly until only single cells were present. From such inocula one could readily propagate new liquid cultures as has been described by Nickell (11) or pipette out on agar in petri plates by the method described by Bergmann (3).

Results & Conclusions

Fresh Weight Increase & Tissue Dissociation in Complex Media. At the outset, it was important to study the growth of callus tissues in liquid media of complex constitution and to use the behavior here as a basis for comparison with growth in synthetic media. For this purpose, liquid media were prepared of the same constitution as that on which the tissue clones were propagated on agar culture, i.e., a coconut-milk medium with 2,4-D and a yeast-extract medium with 2,4-D. For comparison, cultures were grown also on the synthetic medium to be described below. In figures 1a and 1b are shown the growth of Daucus and Convolvulus root callus tissues expressed as total fresh weight and the tissue dissociation in terms of cells per ml over a period of 6 and 4 weeks, respectively.

The two tissues responded differently to the three media. Carrot tissue, propagated on the coconut-milk medium, grew better on the yeast-extract medium for this period and somewhat less on the synthetic medium than on the coconut-milk medium. The development of a population of suspended cells was considerable in all three media and was especially rapid in the two complex media. In Convolvulus increase in fresh weight was greatest on the yeast-extract medium and quite poor on the coconut-milk medium, although cell separation was high in the latter case. Note that Convolvulus grew less rapidly and produced fewer cells in these media than carrot, (a species difference which was consistent throughout these experiments). In the experiments to be described below, as in those shown in figure 1, in-

creases in fresh weight of the cell population and the number of cells in suspension were of the same order of magnitude in the synthetic medium as on the complex media.

Effect of Auxin & Certain Growth Factors on Tissue Weight & Dissociation. In experiments with pea root callus tissue (20) it had been observed that the degree of tissue dissociation depended upon the relative concentration of 2,4-D and of the yeast extract added to the medium. Experiments were set up to see if auxin played a similar important role in the synthetic medium tested here and if any of the other growth factors present in the synthetic medium affected the separation of cells. In figure 2 are the results for carrot (fig 2a) and for Convolvulus (fig 2b). In both tissues, the omission of 2,4-D from the medium had relatively little effect during the short term of these experiments on the increase in fresh weight of the culture for the period tested, but in both cases there was a marked reduction in tissue separation in the absence of 2,4-D. The difference was apparent early in the experiment and became progressively more evident, even while growth continued almost unaffected. The result was the production of solid tissue masses in an essentially clear or non-turbid medium. Continued cultivation of either carrot or Convolvulus cell suspensions in the absence of 2,4-D ended ultimately in the cessation of

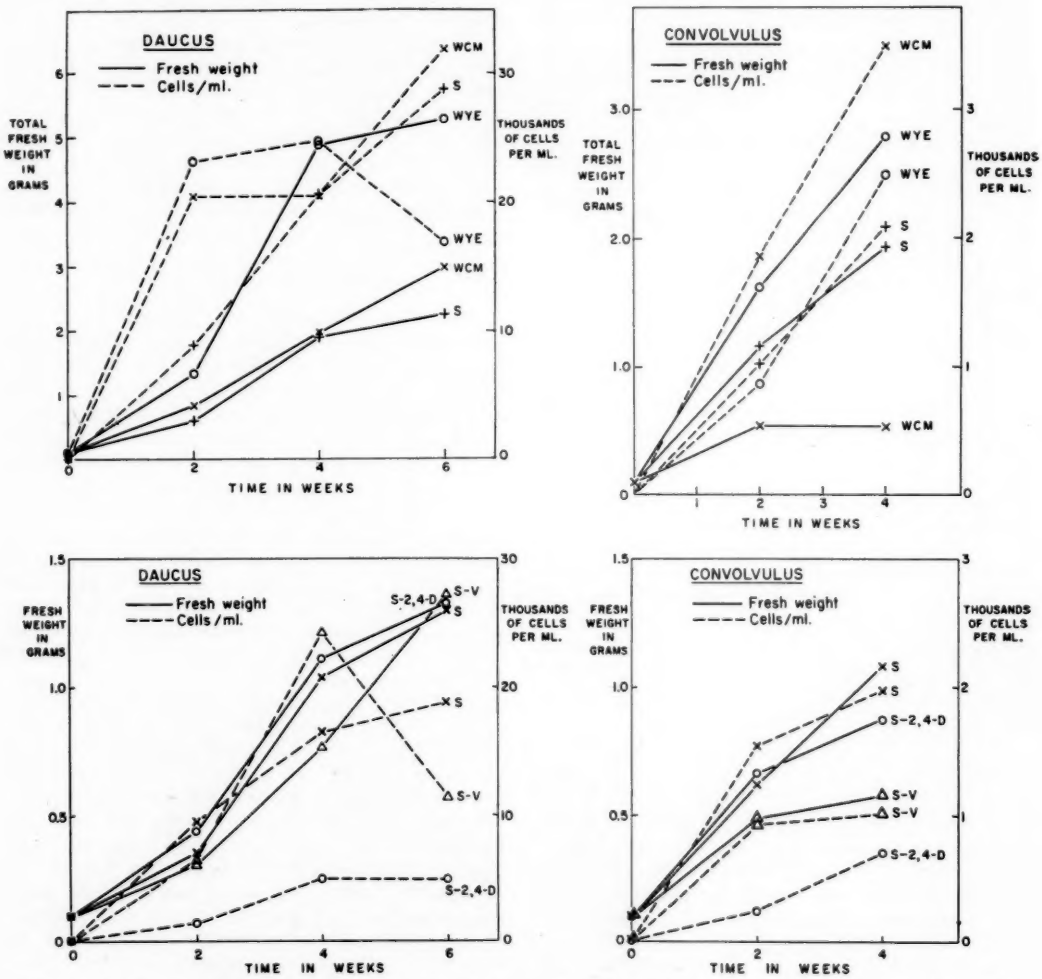

Fig. 1 (*top*). Fresh weight production and cell counts in callus cultures grown in complex liquid media. a (*left*), Daucus; b (*right*), Convolvulus.

Fig. 2 (*bottom*). Fresh weight production and cell counts in callus cultures grown in synthetic liquid medium complete or lacking auxin or vitamins. a (*left*), Daucus; b (*right*), Convolvulus.

growth. Thus the presence of auxin in the medium was essential for continued cellular proliferation but had its most marked effect early in culture on the separation of cells. The optimum auxin concentration to produce maximum separation of tissues into free floating cell suspensions has not been determined.

In the Convolvulus tissue, omission of the vitamin mixture (see Methods, component II) resulted in a general depression of tissue growth and reduced tissue friability. The specific vitamin deficiency producing this depression in Convolvulus tissue growth has not been determined.

In the carrot tissue, omission of all the additional vitamins caused an early depression in fresh weight production which was not apparent in the 6-week cultures. There was a marked increase in tissue friability by the 4th week, but the cell count dropped off markedly at 6 weeks when cell debris was added to fresh weight at the expense of cells in suspension.

The effect (on tissue dissociation) of omitting the vitamins was sufficiently marked in the carrot tissue to warrant a more detailed examination of the effect of omission of each vitamin separately. In figure 3 are presented in a histogram the total fresh weight and number of cells per ml for the carrot tissue grown on seven different media: the complete synthetic medium and the same medium lacking one of the following components: choline HCl, ascorbic acid, biotin, calcium pantothenate, riboflavin and inositol. The data for the three determinations taken at 2 week intervals show progressive changes with time.

Fresh weight increase was consistently best in the complete synthetic medium, a result which has been confirmed in several experiments of this type with several tissues, including Daucus, Convolvulus, and Haplopappus (13). This result does not indicate that the synthetic medium used is the best which can be devised, or even that all the components are essential. It is certain that the synthetic medium can be simplified to meet specific needs of each tissue studied, by omission of one or more growth factors, and perhaps a number of amino acids or amides. It is clear, however, that the synthetic medium reported here goes far toward being a general purpose synthetic medium for cultivation of a variety of higher plant tissues as cell suspensions. That tissue differences exist is clear from the fact that pea root callus tissue does not thrive in this synthetic medium but

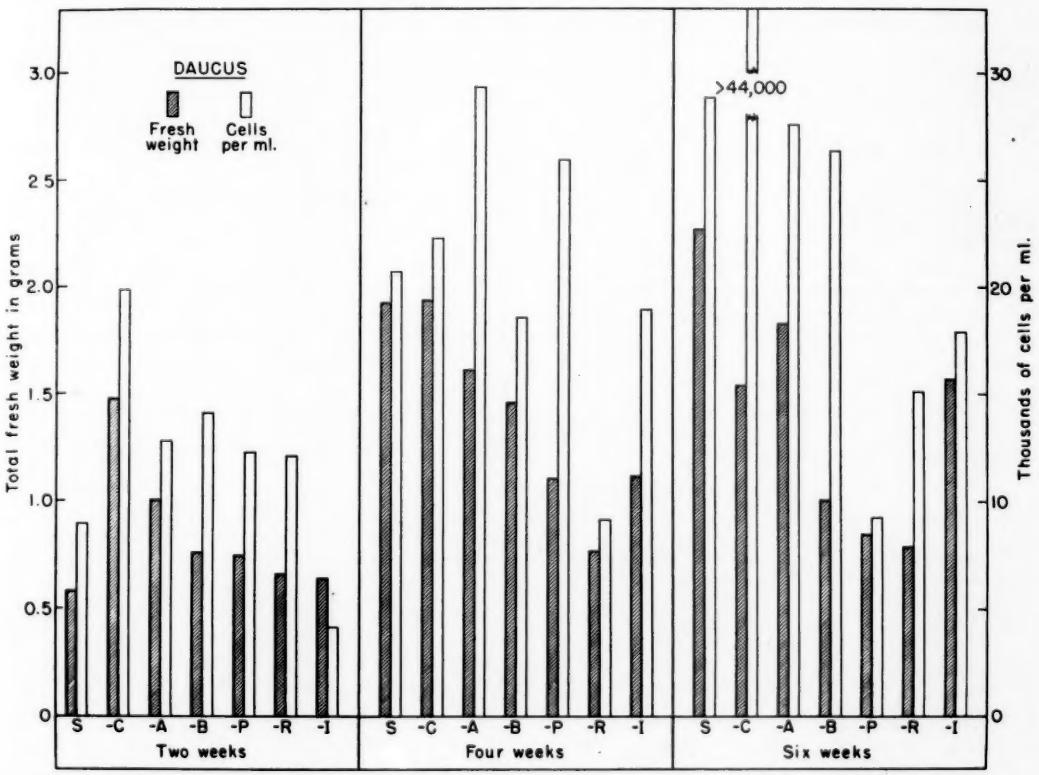

FIG. 3. Histogram showing fresh weight production and cell counts in callus cultures of Daucus, grown in synthetic liquid medium complete or lacking one of the following vitamins: C, choline; A, ascorbic acid; B, biotin; P, calcium pantothenate; R, riboflavin; I, inositol.

does grow rapidly in a simpler synthetic medium (19). Here, the fundamental difference in requirements seems to center around the constitution of the organic nitrogen components, especially the amino acids. In the case of pea root tissue, a single amino acid at toxic concentrations might be the effective inhibitor of growth.

The most striking effect following omission of any single vitamin was the reduction of tissue growth when riboflavin, calcium pantothenate, or biotin were omitted. In the case of riboflavin omission, fresh weight was only about 35% of the control; tissue dissociation was similarly reduced during the 6-week period. Of little or no apparent significance in tissue fresh weight production were the vitamins, choline and ascorbic acid, and of questionable importance was inositol; all of these could be omitted singly without marked effect on the fresh weight. Both choline and ascorbic acid omission affected tissue dissociation significantly, since in the absence of either, the number of cells in suspension was increased.

The omission of single vitamins may have a more marked effect on culture development in suspension than omission of whole groups of growth factors as is apparent in considering the lack of significant decrease in fresh weight production upon omission of all the components of the vitamin mixture (fig 2a). Further detailed analyses of these interactions are clearly necessary to sort the essential vitamins for each tissue. The data in figure 3 suggest that there may well be vitamin requirements for rapid and continued proliferation in cell suspension culture which are not apparent in agar culture of callus tissue masses.

The interpretation of the effect of these omissions is complicated by the fact that, although the tissue weight measurements reflect total increase with time, the cell suspension measurements are not accumulative evaluations since cells sloughed away from tissue pieces do not necessarily continue as viable cells in suspension. They may follow either one of two courses: first, if the medium is unsatisfactory for their survival in isolation from other cells, they die and become cellular debris, contributing to total weight of the culture but disappearing from the cell counts; second, if they are surrounded by an adequate nutritional environment, the isolated single cells in suspension may undergo mitosis and cytokinesis, forming cell pairs. These in turn may divide repeatedly to form multicellular masses with no cell separation. Such cells also disappear from the cell suspension data. Only later are cells sloughed off from these multicellular masses. In no nutrient condition was there evidence that newly-formed cells ever separated immediately. Rather, cell separation appears to be a phenomenon associated with the later stages of tissue proliferation.

The tissue responses to the nutrient media were not always consistent over the period of 6 weeks. For example, the tissue response to omission of choline is striking; after 2 weeks a marked increase in fresh weight occurred compared to the control, with no significant difference from the control at 4 weeks, and about a third less weight than the control at 6 weeks. From data on the incidence of mitosis in these cultures, it was evident that cell multiplication occurred largely in the initial period and had virtually ceased by 2 weeks. Thereafter, tissue weight increases involved deposition of starch and wall materials; this was apparent on cytological observation and was reflected in weight change. A more detailed analysis of medium effects during the early weeks of culture should be carried out.

The average number of cells per milliliter is not necessarily the best index to the effectiveness of the medium in causing cell separation. Another measure is the proportion of the total culture which is in suspension at any time. In table I are summarized the data from several experiments which allow a comparison of the relative effectiveness of different

TABLE I

EFFECT OF DIFFERENT MEDIA ON % OF TOTAL FRESH WEIGHT OF CULTURE IN SUSPENSION & AVERAGE NUMBER OF CELLS PER MILLILITER

MEDIUM	TIME IN WEEKS					
	2		4		6	
	% FR WT SUSPENDED	CELLS/ml + S.E.	% FR WT SUSPENDED	CELLS/ml + S.E.	% FR WT SUSPENDED	CELLS/ml + S.E.
Daucus						
WCM	7.5	20,590 ± 445	2.4	20,470 ± 1,730	3.1	32,330 ± 5,610
WYE	13.3	23,220 ± 3,435	4.7	24,760 ± 4,540	2.4	17,160 ± 1,430
S	12.2	9,240 ± 275	8.5	18,620 ± 1,495	8.8	21,860 ± 4,910
S—2,4–D	2.0	1,590 ± 815	2.3	4,950 ± 1,125	1.4	5,330 ...
Convolvulus						
WCM	5.2	1,870 ± 110	8.1	3,500 ± 20	...	...
WYE	6.5	875 ± 110	3.3	2,530 ± 160	...	...
S	9.1	1,730 ± 190	6.9	1,980 ± 340	...	...
S—2,4–D	1.5	260 ± 170	1.8	710 ± 60	...	...

media in producing growth with cell separation. Tissue friability is expressed here in terms of the per cent of the total fresh weight of the culture which occurs in suspended form as single cells or cell pairs. Data are also given for the average number of cells per milliliter determined by direct count. Some indication of the variability of the cultures is given in the data on cell counts in which the standard errors for these values are given. These are calculated from the data for individual flask values for each sample. The longer the cultures are maintained, the greater is the variation in flasks in any one sample.

In table II are presented representative values for fresh weights and cell counts with calculated standard errors taken from the experiment summarized in figure 3. Decreased variability could be expected with increased replications, but it seems clear from these data that a reliable indication of the relative effect of the various media can be gained from these limited observations.

The most striking fact is that the proportion of the total culture in suspension is greatest early in the experiment and, although the average number of cells per milliliter goes up consistently with time, the relative proportion of the culture in suspension decreases markedly. Omitting 2,4-D results in a drastic reduction of both the number of cells per milliliter and the per cent of fresh weight in suspension; this effect was noted in earlier experiments.

Mitoses occur in single suspended cells and in the tissue clumps during the initial 2 weeks, with a higher rate in the latter component of the culture. Some single cells become clumps; many more mature cells are sloughed off into the medium, and some single cells die and break up. The most rapid growth is clearly in the cellular aggregates; with time a relatively smaller proportion of the total fresh weight of the culture is represented by the cells in suspension.

In general, this same picture of culture development applies to the two tissues. Clearly, in the synthetic medium the proportion of the total fresh weight of the culture which occurs in suspension remains relatively high during the experimental period, suggesting that this medium stimulates a greater continuing separation of cells than the other media stimulate. The ideal situation of cell division followed by cell separation (with up to 100 % of the cells in suspension) is far from achieved. In a number of individual cultures, values were found as high as 18 % of the total fresh weight in suspension, but the mean value for the synthetic medium was about 12 % in the early culture period. It is clear that experiments using cell suspensions for inocula would be best started within the 2 week period of culture and would invoke frequent subculture.

Experiments With Cell Suspensions as Inocula. One great difficulty in these quantitative experiments, as in many experiments with callus tissues, has been to achieve a uniform inoculum from one flask to another. An obvious answer is to use filtered cell suspensions of known cell number. From the results of the above experiments, it is possible to begin to devise the best conditions which will produce large volumes of viable cells in suspension for such inocula. Preliminary experiments in this direction have proved the value and applicability of this approach. Measured volumes [(0.5–5.0 ml) taken from flasks of synthetic medium in which Convolvulus root callus tissues have been propagated for various periods of time] were filtered aseptically and introduced by pipette into fresh flasks of medium or were plated on to agar plates in the manner used for plating bacteria. In the latter case, dishes were then taped shut to avoid desiccation. Estimates of cell numbers were made on separate aliquots of the filtered suspension.

In figure 4 are shown petri plates inoculated in this way. Callus tissue pieces develop from single cells or cell pairs or small cell clumps if provided adequate nutrition (fig 4a), but grow only to a very limited extent on an inadequate medium (fig 4b). No macroscopically visible inocula were present at the beginning of the experiment; thus the limited growth of cell clumps (fig 4b) must be attributed to nutrients carried over by the tissue pieces or present in the inoculum fluid. Cell washing might obviate such carry-over.

Table II

Mean Fresh Weight in Milligrams & Cell Number per Milliliter & Standard Errors for Cultures of Daucus Calculated From Data in Figure 3

Medium		Time in weeks		
		2	4	6
S	Fr wt	0.58 ± 0.02	1.94 ± 0.17	2.27 ± 0.09
	Cells/ml	8,965 ± 475	20,745 ± 1,350	28,830 ± 4,260
S–Choline	Fr wt	1.48 ± 0.14	1.94 ± 0.27	1.54 ± 0.06
	Cells/ml	19,860 ± 2,670	22,325 ± 2,725	44,365 ± 2,070
S–Ascorbic acid	Fr wt	1.02 ± 0.32	1.61 ± 0.27	1.83 ± 0.06
	Cells/ml	12,795 ± 3,500	29,350 ± 1,360	27,600 ± 5,220
S–Riboflavin	Fr wt	0.66 ± 0.13	0.77 ± 0.09	0.79 ± 0.21
	Cells/ml	12,530 ± 480	9,145 ± 2,390	15,090 ± 920

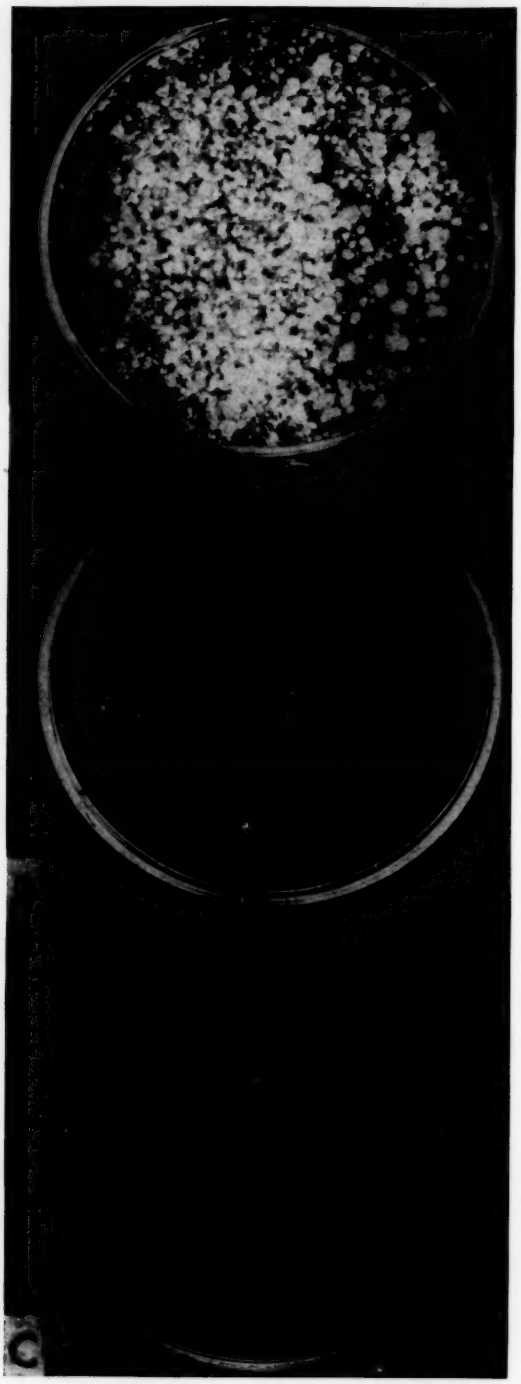

FIG. 4. Ten-centimeter petri plates of nutrient media inoculated with filtered cell suspensions of Convolvulus root callus. a, 2 ml inoculum (ca. 1,500 cells/ml) from 1-week-old culture in synthetic medium, grown 2 months in sealed dish containing yeast-extract-2,4-D medium; b, as in a above but medium lacks yeast-extract and 2,4-D; c, 0.5 ml inoculum (ca. 900 cells/ml) from 1-week-old culture in synthetic medium, grown 2 weeks in sealed dish on yeast-extract-2,4-D medium.

In figure 4c the inoculum was of smaller volume, contained fewer cells, and the culture was photographed after a shorter period of culture growth (2 weeks). Here, discrete callus colonies are evident. Some of these were derived from cell clumps (clumps containing up to 10 cells were present in the inoculum), others were derived from cell pairs or from single cells. Bergmann showed (3) that this method offers a reliable and easy way to produce callus tissue clones of single cell origin, although carry-over of nutrients in the inoculum still makes difficult the assessment of the nutritional requirements of single cells in vitro.

Similar successful experiments have been conducted with Convolvulus cells grown as suspensions in the synthetic liquid medium and then transferred as inocula to agar plates of the same synthetic medium, indicating that this medium adequately provides for the nutrition at least of cell suspensions. Using completely defined synthetic media in this way may be of considerable importance in establishing and maintaining stable callus tissue types in vitro.

DISCUSSION

The importance of cell suspensions for biochemical studies is clearly evident in considering the past successes achieved in understanding intermediary metabolism using bacterial cell suspensions, and in studies of photosynthesis using algal cell suspensions. The availability of large numbers of higher plant cells growing at a rapid rate under defined conditions could contribute significantly to our understanding of problems in higher plant tissue systems where morphology, diffusion gradients, and wound phenomena have combined in the past to limit and frustrate the investigator.

The present work is a necessary step in the direction of obtaining such cellular material for study. The fact that cell suspensions of higher plant tissues can be cultivated in a completely defined synthetic medium under relatively simple conditions means that biochemical studies can now be pursued with these tissues. The evidence is already at hand that cell suspensions represent ideal material for studying especially problems of cellular differentiation.

Considerable improvement can doubtless be made in the techniques described here to increase tissue growth and separation and thus the proportion of the tissue system in suspension, and to simplify the medium itself to meet more precisely the specific requirements of the tissue studied. From the experiments described above, auxin concentration is one of the

critical components of the medium controlling tissue separation. Here is a biochemical problem intimately associated with pectic metabolism and the dissolution of the middle lamella. Cellulose metabolism may also be involved. With a synthetic medium now available, the problem is open to study. Other factors in the medium may also be important in tissue dissociation, notably ascorbic acid or choline. The possible interaction of auxin and ascorbic acid in the control of pectic metabolism in these suspensions is another area of considerable interest.

The present experiments suggest further that the nutrient requirements for continued proliferation of cell suspensions derived from higher plant tissues may be different and more complex than those for the same tissues grown as a multicellular mass on a solidified medium. The data from studies on carrot tissue are not sufficiently extensive to warrant final conclusions, but the evidence suggests that riboflavin certainly, and perhaps also calcium pantothenate and biotin may limit cell suspension proliferation if not provided in the external medium. Such a requirement shown by single isolated cells in suspension but not apparent in tissue grown in multicellular mass would give evidence for the interactions which may occur among cells in intimate contact—and may give us information as to the requirements of single isolated cells grown in vitro.

Summary

A relatively simple technique for the cultivation in a completely defined synthetic medium of cell suspensions derived from higher plant tissues is described. Methods for studying tissue proliferation and tissue separation are outlined and data from experiments with callus tissues from carrot (*Daucus carota* L.) and bindweed (*Convolvulus arvensis* L.) are presented. Tissue growth in terms of fresh weight production is best in complex media but is of the same order of magnitude in the synthetic medium described. Tissue separation into free floating cells is dependent upon the auxin concentration in the medium; in the absence of added auxin, separation is markedly reduced. Evidence is presented for the requirement of certain vitamins by the cell suspension cultures of both Convolvulus and carrot for maximum growth; these are requirements which are not manifest by these tissues when grown on solidified medium. The potential usefulness of the cell suspension technique using synthetic media is discussed.

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HEXITOLS IN COCONUT MILK: THEIR ROLE IN NURTURE OF DIVIDING CELLS

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Coconut milk, the liquid endosperm of the coconut (*Cocos nucifera* L.), has special interest because it will induce otherwise mature, non-growing cells to divide and to grow rapidly (3, 4). Similar properties reside in analogous morphological situations, such as the immature caryopsis of *Zea mays* and the liquid present in immature fruits of Juglans (18) or of Aesculus (12, 24, & also see 25). A nutritive relationship also exists between the female gametophyte, sometimes called endosperm, and the archegonia and embryos of the gymnosperm Ginkgo; extracts of this gametophyte will also induce cells of mature tissue of carrot root to resume active growth (18). Therefore, the fluids that nourish immature embryos seem especially able to induce growth in the mature cells even of some other plants than the ones in which they were laid down. This raises the question whether the behavior of the zygote is due to its special nature or to its nurture by the special fluid contents of the embryo sac, by the substances which are contained in the endosperm, and by other special nutritive organs.

Free cells obtained from mature carrot phloem may be cultured in media which contain coconut milk (21) and may grow and regenerate a complete and mature plant (20). In this respect the free cells imitate the zygote, and the coconut milk its normal nutritional supply. Moreover, as the cells grow and develop, they form structures which are strongly reminiscent of pro-embryonic development (15). Therefore, a full knowledge of the chemical constituents of coconut milk which cause these growth responses would have an important bearing upon many problems of cell growth and cell division. This knowledge would also have important implications for protein synthesis, which is stimulated in quantity and modified in kind during the induction of growth in carrot and potato cells (27).

Following the observations of Blakeslee and van Overbeek (28, 29), work upon the chemical constituents of coconut milk was pursued sporadically in different laboratories (for references see 25). In this laboratory, investigation has been in progress for some years. Clearly, the growth induction which is produced by the coconut milk, over and above the effects due to common nutrients and vitamins, is not a simple effect due to a single substance. On the contrary, it has been emphasized that no substance singly and independently controls cell division (see 25 & references there cited).

In part the effect of coconut milk is non-specific and is replaceable by casein hydrolysate, or by other sources of the reduced nitrogen compounds, from which the cells may synthesize protein more readily than they do from nitrate (13). Even whole coconut milk alone will not trigger the growth of some cells (e.g. potato tuber), for it needs to be supplemented by one of a large array of compounds which are now known to act synergistically with the coconut milk. The substance 2,4-dichlorophenoxyacetic acid (2,4-D), and many of its analogues with different ring configurations or different side chains, can also function in this manner (14, 16, see also 25). Several of the halogen-substituted phenylacetic acids (26) and certain α-substituted propionic acids (14) can also work along with the coconut milk. This paper now designates certain hexitols to be responsible for part of the effect for which coconut milk (or its morphological equivalent) has hitherto been regarded as a specific source.

Early work on the chemical fractionation of coconut milk and similar fluids recently has been reviewed (25). This work encountered the difficulty that, when purified, the isolated substances only expressed their activity in the presence of other sub-fractions from the coconut milk. While this statement still holds true, the work to be described permits the critical identification of at least three of the synergists which contribute to the total growth which is stimulated by whole coconut milk.

MATERIALS & METHODS

Coconut milk was fractionated on ion-exchange resins and by ordinary chemical means which are described below in the appropriate section. Assays for growth-promoting activity were made on standard (3 mg) carrot explants placed aseptically in a basal nutrient medium to which the materials to be tested were added. After 17 days, the final fresh weight was determined; this served as a measure of the relative activity of the materials being tested. Complete details of the method have been published (19).

[1] Received revised manuscript March 6, 1961.

Results

Synthetic ion exchange resins were first applied to the fractionation of coconut milk to test whether or not organic phosphates, which could be bound to a suitable resin (Amberlite IR–45), contributed significantly to the growth induction stimulus. They did not; however, this led to the separation of whole coconut milk into two main parts by passing it over suitable resins. One part seemed to be ionic, usually called "active fraction", and the other part was either very weakly ionic or strictly neutral and is referred to as the "neutral fraction". Although these two fractions, separately, were usually weakly active in the carrot growth assay, they elicited activity which approached that of whole coconut milk when they were tested in appropriate combination. Subsequent experiments showed that the active fraction is also adsorbed by activated charcoal, from which it can be eluted with 50 % aqueous acetic acid followed by 5 % ammonium hydroxide. The chart of figure 1 shows the method which is now used for the separation of

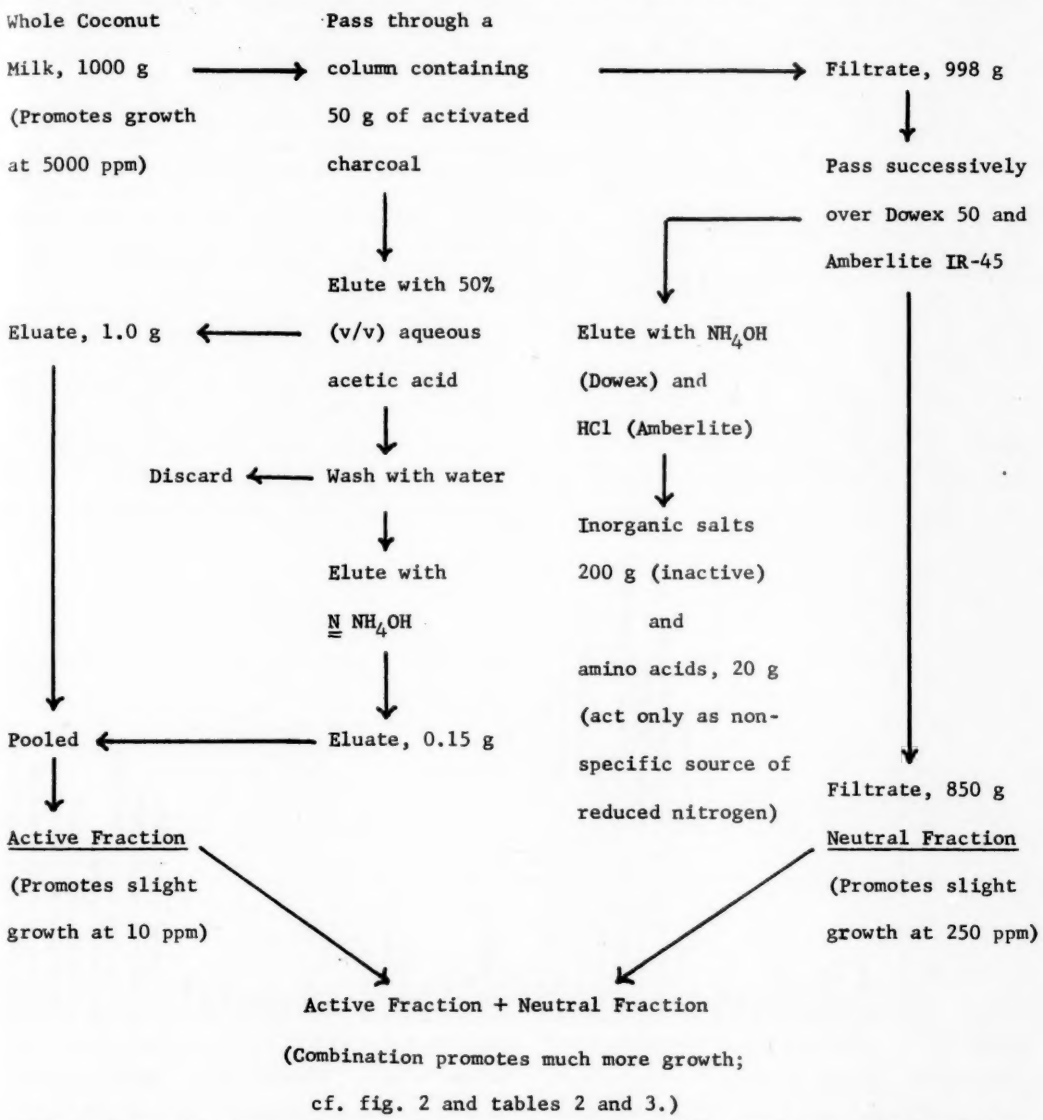
Whole Coconut Milk, 1000 g (Promotes growth at 5000 ppm)
Pass through a column containing 50 g of activated charcoal
Filtrate, 998 g
Pass successively over Dowex 50 and Amberlite IR-45
Elute with 50% (v/v) aqueous acetic acid
Eluate, 1.0 g
Elute with NH_4OH (Dowex) and HCl (Amberlite)
Discard
Wash with water
Inorganic salts 200 g (inactive) and amino acids, 20 g (act only as non-specific source of reduced nitrogen)
Elute with N NH_4OH
Pooled
Eluate, 0.15 g
Filtrate, 850 g
Active Fraction (Promotes slight growth at 10 ppm)
Neutral Fraction (Promotes slight growth at 250 ppm)
Active Fraction + Neutral Fraction
(Combination promotes much more growth; cf. fig. 2 and tables 2 and 3.)

FIG. 1. Preparation of an active fraction and a neutral fraction from whole coconut milk. (Weights as shown indicate the approximate yields of dry matter from 20 liters of whole coconut milk, representing about 1,000 g of dry weight; activities at the concentration indicated refer to the standard carrot assay method.)

coconut milk into these principal fractions. The histograms of figure 2 show that active and neutral fractions supplement each other. When obtained in quantity, each could be used as a supplement to the basal medium when the growth-promoting components of the other were to be detected by the carrot growth assay. The present paper gives an account of the neutral fraction of coconut milk; this fraction is not retained when coconut milk concentrate is successively passed over the resins Dowex 50 and Amberlite IR-45 (see fig 1).

Neutral, or Weakly Ionic, Compounds of Coconut Milk. After the deonization procedures described above, the so-called neutral fraction was evaporated to a thick, dark colored syrup which represented 85% of the dry weight of coconut milk, or 40 to 45 g/liter.

Sorbitol and *scyllo*-inositol (scyllitol) were crystallized, even before they were identified, from the syrup after it had been freed from reducing sugars by passage over a strongly basic resin (Dowex–1). *myo*-Inositol was identified chromatographically and then isolated by a modification of the lead acetate precipitation described by Haas and Hill (5). In table I are listed the criteria upon which these three hexitols were identified.

Subsequently, larger amounts of *scyllo*-inositol were readily crystallized from the crude neutral fraction by evaporating it to a heavy syrup, adding 2 volumes of methanol, and seeding the syrup with crystals of *scyllo*-inositol. After a week at room temperature, 8 g of crude *scyllo*-inositol were obtained in this way from the equivalent of 20 liters of coconut milk. A single recrystallization from water and methanol gave 4.5 g of pure *scyllo*-inositol.

The residue from this larger *scyllo*-inositol isolation was again evaporated to a heavy syrup and an equal volume of dimethylformamide was added. Large quantities of sorbitol crystallized after the mixture was placed in the refrigerator. In this way, 200 g of crude sorbitol, once recrystallized, were obtained from the equivalent of 20 liters of coconut milk. The yield of crystalline sorbitol was estimated at about half the total sorbitol present; therefore, the largest single constituent of coconut milk was one which had previously escaped identification.

The yield of *myo*-inositol was 50 mg from the equivalent of 3 liters of coconut milk. A bioassay, using an inositol-less mutant (no. 37401, kindly supplied by Prof. A. M. Srb) of *Neurospora crassa* (1), indicated that the *myo*-inositol content was approximately 0.25% of the dry weight of coconut milk. *scyllo*-Inositol had no effect on this mutant. The approximate hexitol content of coconut milk is listed in table I.

Subsequent to the work described above, a search of the literature showed that a substance called cocositol was isolated from coconut leaves in 1907 and again from coconut milk by Müller (7); this was later shown to be *scyllo*-inositol (8).

Interactions of Substances or Classes of Compounds Which Produce Growth Response Due

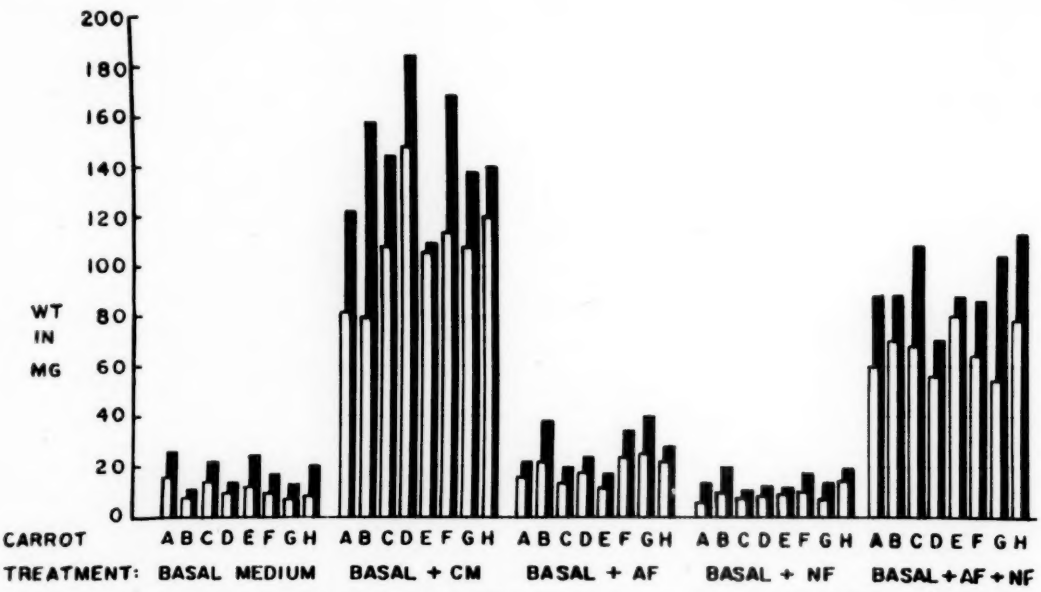
200
180
160
140
120
100
WT IN MG
80
60
40
20
0
CARROT A B C D E F G H A B C D E F G H A B C D E F G H A B C D E F G H A B C D E F G H
TREATMENT: BASAL MEDIUM BASAL + CM BASAL + AF BASAL + NF BASAL + AF + NF

Fig. 2. Growth response of explants from eight different carrot roots to active fraction (AF) and neutral fraction (NF), separately and in combination, grown in the presence (*solid bars*) and the absence (*white bars*) of casein hydrolysate, compared with their growth in basal medium alone and basal medium plus coconut milk (CM). Each bar represents the average final fresh weight of nine replicate cultures after a growth period of 17 days.

TABLE I

EVIDENCE FOR IDENTITY OF 3 HEXITOLS ISOLATED FROM COCONUT MILK

HEXITOL	APPROX. CONC IN COCONUT MILK	ANALYSIS	MELTING PT. (° C)
Sorbitol			
Isolated	15,000 ppm	C, 39.68; H, 7.70; O, 52.78	...
Authentic		C, 39.56; H, 7.69; O, 52.73	...
Sorbitol hexa-acetate			
Prepd. from isolated sample	...	C, 49.53; H, 6.09	103
" " authentic sample	...	C, 49.76; H, 5.96	103
scyllo-Inositol			
Isolated	500 ppm	C, 40.05; H, 6.62; O, 53.16	353
Authentic		C, 40.00; H, 6.67; O, 53.33	353
myo-Inositol			
Isolated	100 ppm	C, 39.95; H, 6.77	227–32
Authentic		C, 40.00; H, 6.67	227–32

In each instance, the isolated and authentic substances were chromatographically identical in several solvents.

TO COCONUT MILK. To reveal the individual and collective effects of various substances, or categories of substances which occur in coconut milk, the substances were supplied alone and in combination to standard carrot explants in factorially designed experiments. Relatively simple statistics permit the total growth which is observed in such an experiment to be partitioned into that growth which is due to lone action of each treatment and that which is due to interactions between the treatments.

The term "interaction", as used here, implies that two or more components work together to produce a response which is significantly different from the growth which would be predicted from the sum of their individual effects. If such a difference is positive, i.e., the growth produced by the combination of treatments is greater than that predicted from their single effects, then the term "synergism" is used to describe the interaction. If such a difference is negative, then it is referred to as "a negative interaction".

Active fraction, which contains the highly specific cell division factors per se, shows statistically significant interactions with A, casein hydrolysate, as a general amino acid source, B, auxin-like substances, including indoleacetic acid and such synthetic sub-

TABLE II

EFFECT OF INDOLEACETIC ACID (IAA), ACTIVE (AF), & NEUTRAL (NF) FRACTIONS FROM COCONUT MILK, SINGLY & IN COMBINATION, ON GROWTH OF CARROT EXPLANTS IN BASAL MEDIUM (BM) CONTAINING CASEIN HYDROLYSATE (CH)*

TREATMENT	(A) SOURCE OF CARROT EXPLANTS								(B) EFFECTS CALCULATED FROM EXPLANTS FROM 8 SOURCES			
	510A	510B	510C	510D	510E	510G	510H	510J	MEAN GROWTH	OBSERVED GROWTH INCREMENT	PREDICTED GROWTH INCREMENT	EFFECT DUE TO INTERACTION
1. Basal+CH	25.1	28.1	10.8	17.7	25.0	20.4	30.1	19.6	22.1	...	...	...
2. " " +IAA (0.5 ppm)	62.8	57.4	29.8	46.4	66.2	46.7	39.7	23.0	46.5	24.4	...	...
3. " " +AF (10 ppm)	18.8	33.5	17.6	61.8	26.6	16.2	23.1	36.7	29.3	7.2	...	...
4. " " +NF (250 ppm)	59.9	55.0	33.5	25.8	64.8	24.6	50.6	22.4	41.7	19.6	...	...
5. " " +AF+NF	59.6	81.1	62.9	84.3	90.5	77.9	99.1	116.2	84.0	61.9	26.8	+35.1**
6. " " +IAA+NF	81.5	72.2	56.4	47.8	88.0	44.5	52.0	36.5	59.9	37.8	44.0	− 6.2**
7. " " +IAA+AF	50.2	88.3	54.5	67.6	93.9	74.4	55.4	121.7	75.8	53.7	31.6	+22.1**
8. " " +IAA+AF+NF	63.8	112.4	56.2	68.2	111.6	86.6	89.3	163.8	94.0	71.9	102.2	−30.3**

* Data are mean final fresh weights (mg) per explant.

In Table IIB, whenever two components are present in the medium, the growth is predicted from the sum of their individual effects. When three additional components are present in the medium (no. 8), the predicted effect is the sum of the effects due to treatments 5, 6, and 7 minus that of treatments 2, 3, and 4. The interaction of the factors is measured by the difference between the observed and the predicted growth.

** Results significant at the 1 % level.

stances as 2,4-D, and C, with the neutral fraction or its individual components.

Table II shows the mean growth obtained in such an experiment which was replicated on explants from eight different carrots. The variable response shown by the explants from the different carrots is quite clear from this table; such variations from root to root have been previously described (23). Although the effects of each treatment on explants from particular carrot roots may be calculated from this table, the following discussion concerns the mean responses for all eight carrot roots as they are stated toward the right of table II. If the mean weight of the basal controls is subtracted from the mean weight obtained in each treatment, then the observed growth increment due to each treatment is derived (table II B).

The data in table II may be analyzed further as in table III. Table III shows, in summary form, the separate effect of each growth factor on the interaction between the other two which are concerned here. Thus, a clear synergism exists between active fraction and neutral fraction in the absence of indoleacetic acid, but if IAA is present, this synergism disappears. Similarly, active fraction and IAA are synergistic if neutral fraction is absent whereas. if neutral fraction is present, no such synergism can be demonstrated. The negative interaction between IAA and neutral fraction, predicted from the above observations, is confirmed by the data. Thus, the neutral fraction and IAA compete, i.e., they show a negative interaction which is accentuated in the presence of the active fraction.

TABLE III

SYNERGISTIC INTERACTIONS BETWEEN GROWTH FACTORS: INFLUENCE OF A 3RD FACTOR ON INTERACTION BETWEEN 2 OTHERS

INTERACTION	INCREMENT IN mg fr wt DUE TO INTERACTION
AF & NF in absence of IAA	+ 35.1
AF & NF in presence of IAA	+ 4.7
AF & IAA in absence of NF	+ 22.1
AF & IAA in presence of NF	− 8.2
NF & IAA in absence of AF	− 6.2
NF & IAA in presence of AF	− 36.5

The effect of the individual treatments as they act alone may be assessed from table II. A more complete statistical analysis of variance, not given here, established that the individual effects of active fraction, neutral fraction, and IAA were all highly significant over the whole experiment.

The complete interpretation of such interactions, synergistic or not, will depend on the eventual elucidation of the biochemical role that each component plays in the living system. The failure of IAA to elicit the expected positive response in the presence of neutral fraction may indicate that, at least in part, these two growth-promoting substances overlap so that the requirement for one is reduced somewhat in the presence of the other. Alternatively, since both IAA and neutral fraction require active fraction for their best response, they may be in competition, at the biochemical level, for the substances which are contained in the active fraction, if all of these components are supplied together.

Although not detailed here, we tested the interaction of IAA and neutral fraction at four levels of active fraction on the growth of explants from four different carrots. If IAA and neutral fraction compete for active fraction, this should be most apparent as a significant negative interaction when the active fraction tends to be limiting. This did not occur. Therefore, the first alternative suggested above is still the preferred one, although it lacks biochemical explanation.

BIOLOGICAL ACTIVITY. The hexitols in coconut milk exert their effect on carrot explants when they are added to the basal medium (which contains salts, sucrose, & certain vitamins), particularly if the active fraction is also supplied. The latter elicits cell division when its active substances are present in the culture medium at concentrations of the order of a few parts per million; the hexitols, however, act at higher concentrations (generally of the order of 50 ppm). Thus a nutrient medium which contains 10 % by volume of whole coconut milk contains approximately 1,500 ppm of sorbitol, a concentration which our assays show are at least ten times greater than that which shows a response with carrot explants. Since these hexitols were added to a medium which was already well supplied with a general carbohydrate source (sucrose), their primary role is not as the source of carbon for the growing tissue. If sorbitol alone is the general source of carbon, the carrot explants cannot grow at all. Table IV contains the results of an experiment in which the growth stimulus was supplied by the active fraction from coconut milk (10 ppm) and by 100 ppm of *myo*-inositol. Of the additional carbon sources thus supplied, sucrose was the best, glucose substituted partially for sucrose, fructose alone was toxic, and sorbitol, mannitol, or dulcitol were without effect. Therefore, sorbitol is not a general carbon source for cultured carrot tissue.

Table V shows the variable responses of explants from different carrot roots to the known components of the neutral fraction. Explants from carrot 498A responded to both *myo*-inositol and *scyllo*-inositol, the greater response being to the former; but in this group of explants no synergistic response between the inositols and (IAA+AF) was noted. This is, however, complicated by the antagonism between IAA and neutral fraction discussed earlier in relation to table II. With explants from root 498D the growth response was not to *myo*-inositol alone by to *myo*-inositol in the presence of (IAA+AF). Here the

TABLE IV

GROWTH OF CARROT EXPLANTS IN MEDIA WHICH CONTAIN VARIOUS CARBOHYDRATES AS SOURCE OF CARBON

CARBOHYDRATE SUPPLIED (1.5 %)	BASAL MEDIUM ONLY SOURCE OF CARROT EXPLANTS		BASAL MEDIUM PLUS ACTIVE FRACTION (10 ppm) & INOSITOL (100 ppm) SOURCE OF CARROT EXPLANTS	
	547A	547B	547A	547B
None	5.6	4.7	7.1	5.5
Sucrose	11.5	9.1	82.9	28.6
Sorbitol	6.1	4.5	5.8	5.0
Glucose	5.0	4.5	45.4	30.8
Fructose*	...	...	...	...
Mannitol	4.8	4.5	7.6	5.3
Dulcitol	5.6	4.9	5.2	5.1

* When fructose is supplied as the sole source of carbon, the medium is toxic after autoclaving. In a comparable experiment where the fructose was filter sterilized, the cultures grew to a final weight which was 70 % of that attained in the controls supplied with sucrose.

magnitude of the interactions was large enough to mask the negative interaction between IAA and the components of neutral fraction. Explants from root 498D did not respond to *scyllo*-inositol and, although the data are not given, explants from roots 498D and 498C did not respond to either of the inositols.

Table VI shows the growth responses of explants from 492B. *scyllo*-Inositol was effective only in the presence of casein hydrolysate and particularly so if active fraction was present as a synergist. Here the data are not complicated by the effect of IAA in the medium.

The results of an experiment designed to replace neutral fraction from coconut milk with a mixture of

TABLE VI

INTERACTING EFFECTS OF CASEIN HYDROLYSATE, ACTIVE FRACTION AT 10 ppm (AF) & SCYLLO-INOSITOL AT 25 ppm ON GROWTH OF CARROT EXPLANTS IN BASAL MEDIUM*

	BASAL MEDIUM		BASAL + CASEIN HYDROLYSATE	
	No AF	+ AF	No AF	+ AF
No *scyllo*-Inositol	9.7	12.7	15.9	29.4
+ *scyllo*-Inositol	9.9	12.6	18.1	48.4

* Data are mean final fresh weight in mg per explant; mean of nine replicates.

TABLE V

EFFECTS OF INOSITOLS & ACTIVE FRACTION WITH INDOLEACETIC ACID (AF+IAA) ON GROWTH OF CARROT EXPLANTS IN BASAL MEDIUM PLUS CASEIN HYDROLYSATE*

TREATMENT	CARROT 498A			CARROT 498B		
	FINAL WT	GROWTH INCREMENT	EFFECT DUE TO INTERACTION	FINAL WT	GROWTH INCREMENT	EFFECT DUE TO INTERACTION
Basal+casein hydrolysate	19.5	...	...	21.8	...	...
" " +*scyllo*-Inositol (25 ppm)	28.9	9.4***	...	21.9	0.1	...
" " +*scyllo*-Inositol (5 ppm)	27.6	8.1***	...	22.8	1.0	...
" " +*myo*-Inositol (25 ppm)	52.0	32.5***	...	20.1	− 0.7	...
" " +*myo*-Inositol (5 ppm)	43.3	23.8***	...	21.2	1.0	...
" " +(AF+IAA)**	49.1	29.6***	...	57.7	37.9	...
" " " +*scyllo*-Inositol (25 ppm)	68.1	48.6	+ 9.6	52.1	30.3	− 7.7
" " " +*scyllo*-Inositol (5 ppm)	57.9	38.4	+ 0.7	51.1	29.3	− 9.6
" " " +*myo*-Inositol (25 ppm)	65.4	45.6	−16.5***	70.2	48.4	+11.2***
" " " +*myo*-Inositol (5 ppm)	61.4	41.9	−11.5***	72.5	50.7	+11.8***
Basal+casein hydrolysate+10 % Coconut milk	124.0	104.5***		95.7	63.9***	

* All data are in mg mean final fresh weight per explant.
** AF used at 10 ppm, IAA at 0.5 ppm.
*** Indicates a result statistically significant at the 1 % level of probability.

carbohydrates are found in table VII. For carrot 510C the response to treatment with this synthetic neutral fraction at 500 ppm was greater than that shown to coconut milk neutral fraction at the same concentration. Explants from carrot 510H grew better on coconut milk neutral fraction, although they still responded to the synthetic mixture. The mixture of carbohydrates used above contained equal amounts of each of the three hexitols of coconut milk and of maltose, mannose, lactose, ribose, rhamnose, erythritol, turanose, trehalose, and glycerol. (In other experiments, all of these, save glycerol, have shown statistically significant stimulatory responses in the presence of the active fraction and inositols.)

In view of the carbohydrate nature of the neutral fraction, a survey was made of readily obtainable carbohydrates to see which were effective in this respect. Out of some 33 carbohydrates tested, those listed above were found to have some statistically significant effect in the presence of both inositols. Until there is actual evidence of the occurrence of these or any other effective carbohydrate in coconut milk, there is no reason to believe that the activity of natural neutral fraction owes anything to compounds other than the two inositols and sorbitol, which do occur in the coconut milk. However, other effective carbohydrates in the neutral fraction of coconut milk may yet be discovered.

Much still remains to be understood, particularly with respect to interactions between different sugars. However, table VII shows that the part of the total effect of coconut milk which is due to the neutral fraction may be replaced by a mixture of pure carbohydrates, and this will, at least for certain groups of carrot explants, substitute for the natural product. In fact, there is no reason to assume that the composition of the neutral fraction of coconut milk is ideal for carrot cells, and, in time, a better mixture of carbohydrates for carrot cells may be developed than that which is found in coconut milk. For example, experiments have already shown that sorbitol is present in coconut milk at a concentration ten times greater than that at which it acts most effectively.

Neutral Fraction From Other Sources of Growth-Promoting Activity. The composition of the neutral fraction from other good sources of the growth-promoting activity toward carrot explants may differ from that which is present in coconut milk. For example, an investigation for hexitols in the liquid from Aesculus fruits produced a somewhat surprising result. When 116 g of this fluid, which had already been through charcoal and freed of certain inorganic substances (equivalent to 145 g dry wt of the whole Aesculus fluid) was de-ionized with Dowex 50 and Amberlite IR-45, only 52 g of organic, neutral fraction remained. This was concentrated to 100 ml; 200 ml of methanol were added, as in the process for preparing *scyllo*-inositol from coconut milk, and crystals appeared instantly. A yield of 14 g of hexitol was thus obtained; however, the melting point did not correspond to *scyllo*-inositol but rather to *myo*-inositol. This product was then identified by comparing the melting points of both the isolate and its acetate with authentic *myo*-inositol and its acetate. The isolate, the *myo*-inositol, and a mixture

Table VII

Comparison of Effects of Neutral Fraction From Coconut Milk, & of Sorbitol, With Mixture* of Hexitols & Sugars on Growth of Carrot Explants in Basal Medium Plus Casein Hydrolysate With & Without Active Fraction

Treatment	Source of carrot tissue					
	Carrot 510C			Carrot 510H		
	Final wt	Growth increment	Effect due to interaction	Final wt	Growth increment	Effect due to interaction
Basal + casein hydrolysate	14.5	...	...	28.8	...	...
" " + neutral fraction (500 ppm)	21.6	7.1†	...	41.0	12.2†	...
" " + mixed hexitols & sugars*	22.4	7.9†	...	30.6	1.8	...
" " + active fraction (10 ppm)	21.4	6.9†	...	24.2	− 4.6	...
" " " + neutral fraction	49.8	35.3	+21.3†	63.7	34.9	+27.3†
" " " + mixed hexitols & sugars	54.6	41.1	+26.3†	39.8	11.0	+13.8†
Basal + casein hydrolysate + sorbitol (100 ppm)	15.9	1.4	...	28.8	0.0	...
" " " + active fraction (10 ppm)	20.8	6.3	− 2.0**	29.5	1.0	+ 5.6** ***
Basal + casein hydrolysate + 10 % coconut milk	59.8	45.3†	...	116.0	87.2†	...

* The mixture consisted of equal parts of the three hexitols in coconut milk plus eight sugars to which carrot tissue had previously shown a significant positive response. The final concentration of the mixture was 500 ppm.

** These results are typical of many. They show for certain carrot roots a small, but significant, positive response to sorbitol in the presence of active fraction, but this is lacking in tissue from other roots.

*** Indicates a result statistically significant at the 5 % level of probability.

† Indicates a result statistically significant at the 1 % level of probability.

of the two all melted in the range 227 to 232° C. Also, the acetate of the isolate, the acetate of authentic *myo*-inositol, and a mixture of the two acetates all melted in the range 220 to 221° C.

At this time neither sorbitol nor *scyllo*-inositol has actually been demonstrated to be present in the Aesculus fluid by its isolation, although either or both may well be present. In contrast to coconut milk, where sorbitol is the predominant hexitol, the liquid of Aesculus contains *myo*-inositol predominantly. In fact, *myo*-inositol represents at least 10 % of the total dry weight, or roughly 25 % of the neutral fraction from Aesculus.

myo-Inositol was also isolated from 15 g of the neutral fraction from *Zea mays*. Following the process which was used to isolate this inositol from Aesculus, 100 mg of crude *myo*-inositol (recrystallized to give 34 mg of pure *myo*-inositol with appropriate melting & mixed melting points) were obtained. Consequently, *myo*-inositol in greater or lesser amounts is present in and is characteristic of three natural sources of the growth promoting activity in question, namely coconut (Cocos), corn (Zea) and horsechestnut (Aesculus). In the last of these sources, *myo*-inositol is present in very large amounts for a substance which is commonly thought to act catalytically or as a vitamin.

Discussion

When the fully autonomous growth of cambium cells gives place to the almost completely quiescent mature phloem cells, losses of metabolic competence occur. These losses may occur at various points which may be relieved in different strains by either exogenous supplies of indoleacetic acid or of the different hexitols, which may be supplied separately or in combination. Indeed, one now can visualize that carrot cell clones with a specific requirement for a given inositol, or for sorbitol, could be established. In fact, as this work progresses, the use of selected clonal strains of cells, cultivated by the methods which have been described (21), promises to be instructive and to permit the complex needed to induce growth to be partitioned into components for the assay of which special strains will be available.

Chemical Complex Required for Growth Induction. To unleash the totipotency which is now seen to reside in even mature phloem parenchyma cells of the carrot root, an array of chemical regulants or stimuli is required. What E. W. Sinnott once referred to as the "primitive built-in goal of growth", which resides in the fertilized egg, is evoked by the nurture which it receives while in the ovule. Whole coconut milk is a substitute for this special nurture, as seen by its successful application to embryo culture, which was first practiced by van Overbeek and Blakeslee (28, 29).

Failure of any of the living cells of the plant body to maintain indefinite growth by cell division can be ascribed to loss of the stimuli in question, to superimposition of an inhibitor in a system of sensitive, poised, chemical regulatory controls, or to simple loss, through irreversible differentiation, of their original capacity to respond to the chemical regulators.

Whereas the inherent capacity of cells to grow may be determined by their genetic constitution, its ultimate expression at any given time is certainly subject to regulation by factors of a non-genetic, or epigenetic, nature in the sense of Waddington (30). For example, both naturally-occurring (17) and exogenously-supplied (22) inhibitors may sensitively inhibit growth of carrot phloem explants that would otherwise be stimulated by coconut milk. In the first case cited, the growth inhibition which is due to the natural product hydroxy-L-proline may be alleviated by simply supplying sufficient L-proline in the growth medium. Identical examples for other growth systems could be cited.

The investigation of coconut milk as a source of these chemical regulators has now progressed to the point where it may be briefly summarized. In addition to the usual inorganic nutrients, main sources of carbon, and common vitamins, this complex of additional requirements for growth may be divided into three component parts as follows:

I. Nitrogenous Component. This consists of reduced nitrogen compounds in the form of amino acids and their amides. The requirement for this component is customarily met by adding enzymic casein hydrolysate, individual amino acids, or even urea or ammonia to the nutrient medium. These substances supply the greater need of growing carrot tissue for organic nitrogen compounds than that which can be met from nitrate alone.

II. Neutral Component. This component consists of a variety of carbohydrates, which are not required primarily as sources of carbon. They do, however, permit the cells to respond to other growth-inducing compounds. While all the substances in this category may not yet be completely characterized, *myo*-inositol, *scyllo*-inositol, and sorbitol are prominent.

III. Active Component. These substances interact with the other parts of the system, and they include representatives of the two main classes of known growth regulators for plant cells. The auxins are best known for their effect on cell enlargement, and they include the naturally-occurring substances like indoleacetic acid, or such synthetic substances as 2,4-dichlorophenoxyacetic acid. By contrast, the class of regulators which specifically stimulate cell division and which are found in that fraction of coconut milk which has been designated "active fraction" is still not completely known. Equivalent active fractions are readily obtainable from immature corn (*Zea mays*) extracts or from the fluid from young Aesculus fruits.

Hitherto, an obstacle has been that substances in

this class, even when isolated from relatively large amounts of material, have been obtained in too small amount for satisfactory purification and identification. Now, however, in the secure knowledge that the constituents of coconut milk (which comprise two of the three main components of coconut milk) are sufficiently defined to be furnished directly, a massive attack upon the constituents of the active component may be made.

The prominence given here to the hexitols as constituents of coconut milk is suggestive from the following points of view. First, inositols, free and as phosphates, have long been known to be prominent in endosperms. Second, Braun (2) found that *myo*-inositol, along with an auxin-like compound, was required for the growth in culture of tissue of crown gall origin. A different type of proliferative growth, namely the development of nodules on legumes, is also enhanced by the presence of *myo*-inositol in the culture medium. This was demonstrated in root cultures by Raggio, Raggio, and Burris (10). Finally, there has developed the empirical use of coconut milk as an extender for the fluid in which bull sperm are maintained for artificial insemination purposes (9). The unexpected effect of coconut milk in the latter case serves as a reminder that seminal fluid always appears to contain *myo*-inositol and sorbitol; the latter compound occupies a key role in the respiration of the sperm (6).

The exact role of the hexitols in the carrot tissue remains to be determined. The solution of these latter problems awaits the outcome of experiments with C^{14}-labelled hexitols which are now in progress.

Recommendations are continually being made concerning the composition of so-called synthetic media for the growth of plant tissue in culture; a particular example is the medium suggested by Reinert (11) for the growth of carrot tissue. In this example, the growth in question is the continued growth of long established, or habituated, tissue in contrast to the induction of growth in the hitherto quiescent cells of carrot phloem, which is most in question in this paper. Despite the many constituents of this supplemented basal medium of White, as suggested by Reinert (11), even the habituated or cloned carrot cultures grew better in the presence of coconut milk. However, many of the main components of coconut milk are in fact represented in the medium suggested by Reinert, as follows: The neutral component is partially represented by inositol, in Reinert's section B; the nitrogenous component by a long list of 18 amino acids, which in our work are replaced by casein hydrolysate or by certain simple forms of reduced nitrogen (13); the more biologically active component of coconut milk is partially represented by the added auxin (IAA or 2,4-D). Thus the medium recommended by Reinert contains substances which may, at least in part, play the role of the three main components of coconut milk, whose interactions have been studied in this paper, but the active fraction which specifically promotes cell division is conspicuously lacking, and the possibility of overlapping —or even opposed effects—between IAA and the constituents of neutral fraction (e.g. inositol) was not appreciated. Therefore, the Reinert medium, though apparently defined, is incomplete for maximum growth of carrot tissue. To the extent that the Reinert medium adds so large a number of substances (36 substances) both organic and inorganic, many of which may be dispensable and from which it is difficult to exclude all impurities, it does not define the growth requirements of the tissue as strictly as may seem to be the case.

Summary

The role of liquid endosperms, particularly coconut milk, depends on a delicately balanced complex of interacting components. These include a source of reduced nitrogen which may be replaced by casein hydrolysate, a source of cell division factors supplied as a concentrate which may be prepared by adsorption from coconut milk on activated charcoal and subsequent elution, and a neutral fraction which is prepared by de-ionization of whole coconut milk. The growth data obtained by the use of the carrot tissue culture system indicate that the active fraction, which is required in concentrations of the order of a few parts per million, acts synergistically with the neutral fraction and with indoleacetic acid. Neutral fraction and IAA, acting together, usually produced less growth than that which was to be expected if their effects were additive. This is interpreted to mean that, in at least part of the growth response, neutral fraction and IAA produce overlapping or competing effects.

myo-Inositol, *scyllo*-inositol, and sorbitol have been isolated in crystalline form from coconut milk neutral fraction. They have been critically identified and shown to contribute most of the growth-promoting potential of this fraction. All the evidence assigns to *myo*-inositol the key role in this fraction, with lesser effects being due to sorbitol, to *scyllo*-inositol, and possibly to compounds in the neutral fraction which still remain to be identified. In addition, *myo*-inositol has been isolated from corn in the milk stage and from the fluid from Aesculus fruits, in which it represents 10 % of the dry weight.

Of the various components of coconut milk which promote the growth of carrot tissue explants, only the active fraction complex remains to be more completely identified.

Acknowledgments

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MASS ADAPTATION TO SELENOMETHIONINE IN POPULATIONS OF CHLORELLA VULGARIS [1, 2, 3]

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Permanent transformations in populations of microorganisms are most frequently brought about by environmental selection of randomly occurring mutants. Changes that can be environmentally induced in all or most cells of a population and retained in environments that are non-inducing have also been reported. One instance is the alteration in serotype of protozoa such as paramecium, occasioned by a variety of treatments (1, 11, 12). Examples of bacterial resistance to drugs and other chemicals have recently been discussed in a symposium on adaptation in microorganisms (5). The precise biochemical mechanisms for these transformations are obscure, but there is general agreement that they involve shifts to new, heritable, steady state levels of cytoplasmic metabolic pathways within the genetic framework of the cell. In terms of known biochemical events, the clearest example of induction and maintenance of a new steady state is that of the adaptive β-galactoside enzyme system in populations of *Escherichia coli*. Cells induced to synthesize the β-galactoside permease and enzyme, when transferred to a new situation under which the parent phenotype remains non-induced, will maintain their adaptation indefinitely (2, 3, 4, 9). In these experiments, however, traces of the inducing agent were necessary in order for the maintenance effect to continue.

Earlier work with *Chlorella vulgaris* (15) indicated that populations of this organism were able to adapt to the presence of the antimetabolite selenomethionine, and that the change, despite two subcultures (approx 20 generations), was maintained in medium free of the analogue. This adaptation has now been studied in greater detail. The present paper will describe the morphological events that occur during the adaptation as well as evidence that a transformation of all cells takes place rather than a selection of mutants. The following paper (17) will give data to show that the transformation can be maintained for at least 200 generations in the complete absence of the analogue and that reversal to sensitivity can be attained by specific physiological treatments.

MATERIALS & METHODS

The strain of *Chlorella vulgaris* B. used (14), the composition and preparation of liquid media, the preparation of inocula, and the counting technique have been described earlier (14). Cultural environment such as light intensity, temperature, and rate of flow and composition of the gas mixture were as noted in a later paper (16). For the plating experiments the solid medium consisted of the inorganic nutrient solution, 1 % glucose and 3 % agar. In each petri dish were placed the required aliquot of selenomethionine solution, sterilized by sintered glass filtration, water, if necessary, to give a total of 1 ml, 1 ml of the appropriate number of washed cells, and 20 ml of the melted agar. Each plate was swirled repeatedly to ensure a thorough distribution of the cells and selenomethionine. The petri dishes were wrapped in Saran wrap (Dow Chemical Co., Midland, Mich.) to reduce desiccation, and they were incubated at 24 to 25° C under a bank of daylight fluorescent lights at a light intensity of about 175 to 200 foot candles, unless otherwise noted. A colony counter equipped with a supplementary lens to give a magnification of two to three was used to examine the plates.

RESULTS

POPULATION CHANGES IN LIQUID MEDIUM. Previous observations (15) of *Chlorella vulgaris* cells whose division but not growth had been prevented by selenomethionine, provided a clue which suggested that all or a good many of the inoculum cells were able to adapt to the presence of the analogue: autospores could be clearly seen within many of the giants toward the end of the uncoupled growth interval as well as during the subsequent exponential phase of the growth curve. The enlarged cells failed to disappear completely from the medium, however, even when the stationary period had been reached. The origin and number of giants remaining would therefore have an important bearing on any interpretation given to the nature of the adaptation of the culture, for in spite of the autospores within the giants, there

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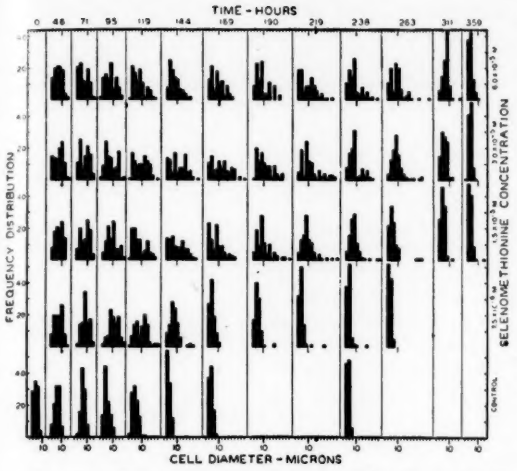


FIG. 1. Changes in diameter of *Chlorella vulgaris* cells during culture in the presence of selenomethionine.

was no assurance that the daughter cells to which they gave rise were viable.

To characterize more quantitatively the changes in numbers and diameters of the giants, periodic measurements were made of 100 cells from each of a series of cultures that contained selenomethionine in graded concentrations. When population densities exceeded 100 cells in the hemacytometer field, separate counts were made for total number and for cells larger than 10 μ in diameter. Many of the enlarged cells were spherical; others were oval, pear-shaped, or irregular in outline. The longest axis of these cells was measured.

Figure 1 illustrates the shifts in cell diameter that took place in the control culture and at the different levels of analogue. Normal populations, derived from an agar slant, initially underwent a general size increase, but seldom were there cells larger than 10 μ in diameter. At the start of the exponential phase (fig 2) the trend toward a smaller size range began, and the population stabilized itself at about three to six μ. Smaller cells were rare. Packed volume and dry weight measurements made previously (15, 16) showed similar correlations. Such changes are also typical of *Chlorella ellipsoidea* (18).

In media that contained selenomethionine approximately 50 % of the inoculum cells grew to more than 10 μ in diameter. At the lowest level of analogue (7.5×10^{-6} M), in which uncoupled growth lasted for only about 90 hours (fig 2), the maximum size observed was only about 20 μ and with such cells few and far between. At the three higher concentrations, algae as large as 25 μ were found more frequently. This diameter, which is about two and one-half times that of the largest normal cell, represents a 16-fold increase in cell volume, as well as enlargement of the chloroplast.

Although the amount of selenomethionine had been progressively raised by double increments, proportional changes in the duration of uncoupled growth were not evident. It would seem, to judge from the actual time lapses (fig 2) and the accompanying changes in giant cell number and size (fig 1), that a limit was approached beyond which additional selenomethionine had no further effect. Higher concentrations have not been tested in liquid media.

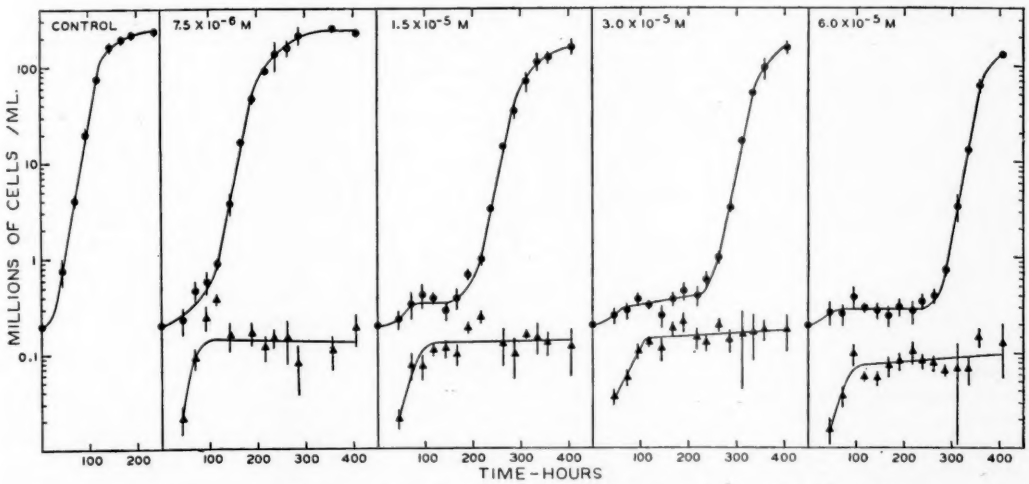
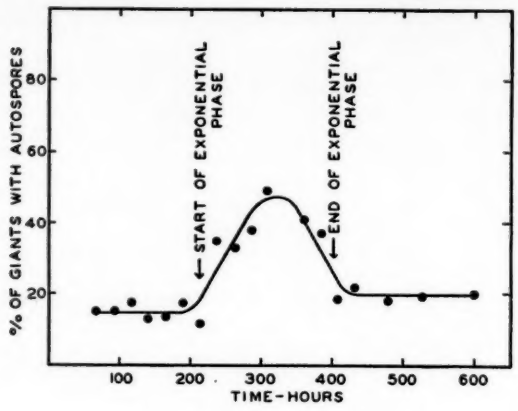


FIG. 2. Growth curves for *Chlorella vulgaris* at several concentrations of selenomethionine. *Upper curves*: ●, total population. *Lower curves*: ▲, cells larger than 10 μ in diameter. Each point plotted with its 95 % confidence interval (standard error × t value).



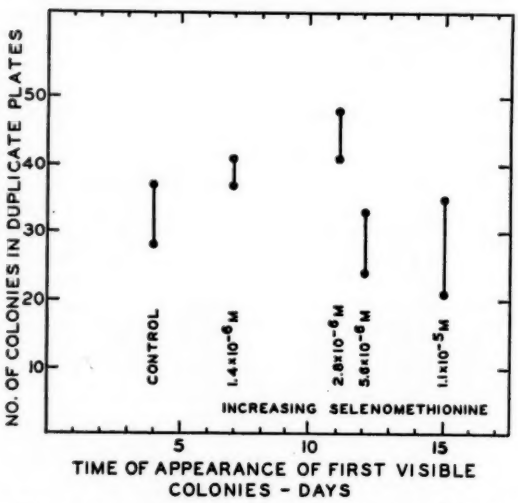


FIG. 3 (*top*). Change in proportion of giant *Chlorella vulgaris* cells containing autospores during culture in 3.0 $\times$ 10^{-5} M selenomethionine (see fig 2 for corresponding growth curve).

FIG. 4 (*bottom*). Time of initial appearance and final number of *Chlorella vulgaris* colonies at increasing concentrations of selenomethionine. Plates incubated at room temperature, under a light intensity of 50 ft-c, for 30 days.

The histograms of figure 1 fail to show giant cells during the late stages of the growth curve. These cells were nonetheless present but in such low frequency that the probability of their being counted among the 100 cells in the hemacytometer field was exceedingly slight. The separate counts made for giant cells are illustrated in the lower curves of figure 2. The giants increased and appear to have remained at a number approximately half the total population density that was maintained during the uncoupled growth period, even at the lowest concentration of selenomethionine. One might conclude, on the basis of the fairly uniform giant count throughout most of the uncoupled phase as well as during the later stages of the growth curve, that a cell, once it enlarged, remained so; the resumption in divisions, therefore, would have had to stem from the normal-sized cells. This interpretation is belied, however, by the observation that the number of giants with autospores rose significantly during the acceleration and exponential phases, and then dropped during the stationary phase (fig 3). The plating experiments described later will also show that each giant is potentially capable of giving rise to viable daughter cells.

That the newly arisen population was indeed adapted is seen from the data of table I. Two passages in the presence of selenomethionine sufficed to create a population that no longer underwent an extended period of uncoupled growth during subsequent transfers with the selenium analogue. Exponential rates also approached those of control cultures. Since approximately ten generations result during each subculture, the change in the population to maximum adaptation must have occurred somewhere between the 10th and 20th generations. Several features, however, differentiated adapted cultures from normal ones. The lag phase, though short, was indicated to be significantly longer, statistically, at the 95 % level, than the lag of controls. More important, perhaps, the enlarged cells never completely disappeared. Table II shows that for the first few passages these cells persisted in numbers comparable to those in cultures exposed only once to selenomethionine. Eventually, after additional subcultures, the numbers of giants declined to very low frequencies which, however, were much higher than the numbers of giants/ml originally present at inoculation. Neither the occurrence of autospores nor size distributions were measured, but it was apparent that the diameters of the enlarged cells ranged between only 10 and 15 μ; the cells rarely attained the extreme giant size prevalent in one-time uncoupled cultures.

PLATING EXPERIMENTS. In order to estimate the proportion of inoculum cells able to adapt, as well as to determine whether daughter cells that sprang from giants were viable, a series of agar plates with graded amounts of analogue were inoculated with small numbers of cells. Figure 4 shows that, within the concentration range tested, each increment of selenomethionine caused a corresponding delay in the appearance of colonies, and that the final number of colonies, after 30 days of incubation, equaled that in control plates. In other experiments similar results were obtained at a concentration of 1.4 $\times$ 10^{-5} M, but a level as high as 1.8 $\times$ 10^{-5} M yielded lower proportions of colonies, even after periods of incubation as long as 8 weeks. The range between 1.4 and 1.8 $\times$ 10^{-5} M, therefore seems to be critical insofar as development of the maximum number of colonies is concerned. In this connection it should be noted that

TABLE I

SERIAL SUBCULTURE OF CHLORELLA VULGARIS IN PRESENCE OF 3.0×10^{-5} M SELENOMETHIONINE

ADAPTED SERIES			CONTROLS			
			+ SELENOMETHIONINE		UNTREATED	
PASSAGES IN SELENOMETHIONINE*	UNCOUPLED GROWTH (hr)	EXPONENTIAL GROWTH CONSTANT	UNCOUPLED GROWTH (hr)	EXPONENTIAL GROWTH CONSTANT	DURATION OF LAG (hr)	EXPONENTIAL GROWTH CONSTANT
1	104	0.038			...	...
2	69	0.050	107	0.037	18	0.066
3	19	0.050	120	0.044	7	0.064
4	24	0.057	145**	**	7	0.058
5	25	0.061	161	0.049	15	0.057
6	27	0.066	125**	**	19	0.072
7	21	0.066	145**	**	19	0.065
8	18	0.063	130**	**	7	0.061
9	21	0.066	165**	**	14	0.064
	22 ± 3.0***	0.061 ± 0.006***			13 ± 5.1***	0.063 ± 0.005***

* Subcultures made from cultures that had reached their stationary phase of growth.
** Too few points to calculate slope of exponential phase of growth; duration of uncoupled growth phase estimated.
*** Mean & 95 % confidence intervals based on last seven passages.

in liquid cultures divisions resumed at as high a concentration as 6.0×10^{-5} M.

The ability of each cell to eventually adapt and multiply is further borne out by the data of table III, in which serial dilutions of cells were exposed to one concentration of selenomethionine. At extremely high densities of about 3×10^6 or more cells per plate, greening of the plates occurred from one to several days after the comparable controls. But with lower cell densities colonies became apparent to the eye only after a much greater delay. The final numbers of colonies in treated plates, even at the highest cell dilutions, equaled those in control plates, some of which developed only one colony.

Though all cells of the inoculum could adapt, it became apparent that their ability to do so was not synchronous. Daily microscopic examinations of plates heavily seeded with 5×10^5 cells established such a heterogeneous response (table IV). One hundred cells were chosen at random in marked fields, and the lineage of most of them was followed until colonies became visible to the eye. Two days after inoculation the majority of untreated cells had divided into clones of eight cells. By the 3rd day 91 % of the clones consisted of more than eight cells, and after 5 days some colonies were visible to the eye. By contrast, single cells in treated plates persisted for a much longer time. Some of these cells reached a

TABLE II

OCCURRENCE OF GIANT CELLS DURING SERIAL SUBCULTURE OF CHLORELLA VULGARIS IN PRESENCE OF 3.0×10^{-5} M SELENOMETHIONINE

ADAPTED SERIES				SELENOMETHIONINE CONTROLS	
PASSAGES IN SELENOMETHIONINE	GIANTS AT END OF SUBCULTURE	INCREASE IN GIANTS*	TOTAL CELLS AT END OF SUBCULTURE	GIANTS AT END OF SUBCULTURE	TOTAL CELLS AT END OF SUBCULTURE
	10^5 cells/ml	%	10^6 cells/ml	10^5 cells/ml	10^6 cells/ml
1	1.0		178.0		
2	3.0	2650	113.5	3.1	23.8
3	2.4	450	221.6	4.4	31.5
4	1.7	780	130.2	1.4	0.6
5	0.3	115	258.0	1.9	30.1
6	1.0	4300	177.0	2.0	1.1
7	0.5	440	187.6	2.1	1.0
8	0.3	565	267.0	2.3	1.2
9	0.3	1335	229.0	1.3	0.6

* Inoculum size at each subculture equal to 2.0×10^5 cells/ml. Giants in inoculum calculated from adapted population of previous passage from which inoculum was taken.

TABLE III

DEVELOPMENT OF CHLORELLA VULGARIS COLONIES IN PRESENCE OF 1.4 × 10^{-5} M SELENOMETHIONINE*

TIME OF APPEARANCE OF FIRST VISIBLE COLONIES (DAYS AFTER INOCULATION)		FINAL NUMBER OF COLONIES		INCUBATION TIME (DAYS)
UNTREATED CONTROLS	+ SELENOMETHIONINE	UNTREATED CONTROLS	+ SELENOMETHIONINE	
5	10,9	93	84,110	
5,5,6,5,5	10,11,10,10,11,12	5,8,9,10,16	2,5,7,8,10,12	
8,12,...	14,14,14,14,17,...	1,1,0,0,0	1,1,1,1,1,0	23
4,4	10,10	145,165	94,133	
4,4	10,10	27,35	10,17	
4,4	10,...	4,4	3,0	26
6	11	134	112	48
6,6	15,13	54,65	50,63	31

* Figures, for the most part, are for duplicate or more plates.

diameter three times greater than that of the largest normal cells. Groups of two, three, four, or more cells could be seen with increasing frequency during the incubation period, and by the 12th day a few visible colonies had appeared. At the close of the experiment, when the plates had become dark green and many of the larger colonies had begun to obscure the smaller clones, single, enlarged cells as well as an array of clones of varying cell number could still be microscopically detected.

Colonies that eventually emerged in selenomethionine-containing plates were morphologically indistinguishable from normal colonies. Several of the colonies were fished and their cells tested, by plating, for adaptation toward the analogue. Table V summarizes the times when clones derived from cells of a one-time adapted colony first became visible. It is evident that these cells could develop into colonies in a significantly shorter time than cells exposed to selenomethionine only once. The slight delay between the appearance of control and adapted colonies, comparable to the slightly longer lag in liquid cultures of adapted populations (table I), was not shortened by several subcultures with the analogue.

Others of the one-time exposed colonies were not maximally adapted, however, and experiments are in

TABLE IV

MICROSCOPIC DEVELOPMENT OF CHLORELLA VULGARIS CLONES IN PRESENCE OF 1.4 × 10^{-5} M SELENOMETHIONINE

DAYS AFTER INOCULATION	UNTREATED CONTROL						+ SELENOMETHIONINE					
	CELLS PER CLONE						CELLS PER CLONE					
	1	2	3–4	5–7	8	>8*	1	2	3–4	5–7	8	>8*
	Number of clones						*Number of clones*					
1	80	1	7	11	1		96	1	2			
2	15	5	16	2	56	6	93		6	1		
3			4		5	91	78	13		4	5	
4	4				2	94	42	6	31		20	1
5						100**	19	8	40	9	20	4
6							24	9	28	16	17	6
7							21	17	31	12	9	10
9							26	8	36	13	8	9
10							18	7	27	22	16	10
11							19	6	20	23	13	19
12							22	6	18	13	20	21**
14							18	7	12	14	18	31
16							17	5	11	10	11	46
18							17	5	9	8	5	56

* Cell numbers in clones that consisted of more than eight cells were difficult to estimate.
** Several colonies visible to the eye.

TABLE V

SERIAL PASSAGE OF CHLORELLA VULGARIS CLONES IN PRESENCE OF 1.4×10^{-5} M SELENOMETHIONINE*

TIME OF APPEARANCE OF 1ST VISIBLE COLONIES			
ADAPTED SERIES		CONTROLS	
		+ SELENOMETHIONINE,	UNTREATED
NO. OF PASSAGES	DAYS AFTER INOCULATION	DAYS AFTER INOCULATION	DAYS AFTER INOCULATION
1	15,15		6,6
2	7,8,9	13,15	6,6
3	7,7	15,18	5,5
4	7,8	11,...	6,6
5	8,8	11,11	6,7

* Figures are for duplicate or triplicate plates.

progress to determine the extent of adaptation and the frequency with which these colonies occur.

DISCUSSION

A number of arguments can be mustered to show that adaptation of *Chlorella vulgaris* populations to selenomethionine in liquid media involves a transformation of each cell rather than selection of mutants from the inoculum. Most convincing are the data of the plating experiments. Over a wide range of inoculum sizes, the colonies that eventually develop in the presence of selenomethionine are equal in number to the colonies that appear in control plates. Moreover, under the microscope each cell can be seen to undergo a period of enlargement before the resumption of divisions which lead to colonies. Thus, the possibility that visible colonies were derived from mutants that arose in microcolonies (13) is eliminated. The resulting colonies of *Chlorella vulgaris* are morphologically indistinguishable from untreated colonies, and some are maximally resistant to selenomethionine after a single plating in its presence.

It should be mentioned that ever since the inception of these experiments, a total of approximately 6.5 to 7.0×10^7 cells has been plated in the presence of selenomethionine, and, except at extremely high cell densities or highly diluted concentrations of the analogue, never have colonies appeared at the same time as the untreated controls. Mutants, completely resistant to any one concentration of selenomethionine, if they exist, would therefore be of very low frequency.

In liquid culture, the fact that the duration of uncoupled growth is controlled by the concentration of selenomethionine may also be cited as evidence for a mass adaptation. Were these effects due to selection of mutants there would have had to exist a graded series, those with the least resistance being present in the largest numbers. The apparent limit to the duration of uncoupled growth in liquid culture, as the selenomethionine was increased, may represent the permeability bounds of the cells toward the analogue molecule under these growth conditions.

Another sign that each cell in liquid culture can adapt is the increased number of giants with daughters at the time that the culture resumes multiplication. The persistence of giants through the stationary phase, however, seems to contradict this interpretation. The apparent inconsistency can be resolved by taking into consideration the heterogeneous response of the cells to selenomethionine, as seen from the plating experiments as well as from the non-synchronized cell enlargement in liquid culture. Concomitant with the enlargement of about 50 % of the inoculum cells in liquid media, and during the subsequent exponential increase in cell number, the smaller cells undoubtedly also enlarge, but by the time they reach a biochemical state favorable for division, the medium has aged as a result of cellular activities so that the later-formed giants remain enlarged. On agar plates of low cell density aging of the medium due to cellular activities is not a problem, and all cells can enlarge, then divide and develop into colonies. In crowded plates, however, aging of the medium is a factor to be considered and probably accounts for the persistence of enlarged, single cells after most of the cells have multiplied into colonies. The heterogeneous response in both liquid and solid media indicates a mixture of inoculum cells of varying susceptibility to selenomethionine, with some cells able to develop into giants sooner than others. The existence of a sequential cycle of developmental stages, each distinguished by its morphological, physiological, and biochemical features, has recently been established by Hase and coworkers (6, 7) in another species, *Chlorella ellipsoidea*. Such a cycle may exist in *Chlorella vulgaris* and account for the heterogeneous response. On the other hand each cell, regardless of the stage at which it is first exposed to selenomethionine, could differ in its susceptibility.

There seems also to be a non-uniform transmission of the adaptation to the progeny, for enlarged cells continued to develop in liquid media even after the extended phase of uncoupled growth had been

eliminated by subculture with the growth uncoupling agent. Table II shows that during each subculture, giants increased 100 to several thousand times over the number of giants originally present in the inoculum. The gradual decline in frequency of giants may mean that, whatever the mechanism of transmission, the adaptation was "built" into the cells during the nine serial passages, so that fewer and fewer susceptible cells eventually arose. An irregular distribution of the adaptive mechanism may also be inferred from the fact that the autospores within the giants frequently were unequal in size. If this is the situation then some of the giants that remained during the stationary phase of one-time adapted cultures may have arisen secondarily from daughter cells derived from the original enlarged inoculum cells.

Population changes in many respects similar to the ones described here have been reported for *Ankistrodesmus braunii* during adaptation to rubidium (8, 10). In liquid cultures that contained RbCl in place of KCl, cells of this alga underwent atypical enlargement for an extended time period, followed by a resumption of divisions and an increase in the proportion of typical-shaped cells. The resulting cells grew at normal rates after transfer to an identical medium, and the adaptation was retained after 105 generations away from Rb. On Rb agar plates freed from K, however, fully adapted clones were never obtained, and only a few partially adapted colonies could be isolated. The assumption was therefore made that at least two stepwise mutations were responsible for the complete adaptation in liquid media.

Unlike the situation reported for Ankistrodesmus, resistance to the growth-uncoupling effect of selenomethionine appears to be induced in each cell of *Chlorella vulgaris*. In this respect the change resembles the well-known adaptive phenomena of bacteria and yeasts in which enzymes, originally undetectable, appear in each cell after introduction of certain chemicals into the environment. In these cases, however, omission of the chemical leads to a rapid dilution of the enzyme as the cells divide, and reversion to the original state is reached after relatively few divisions. With the *C. vulgaris*-selenomethionine system, as will be described in the following paper (17), the transformation can be retained for more than 200 generations in the complete absence of the causative agent, with reversal possible, however, by subculture of the adapted algal cells in either D- or L-methionine, or under conditions of sulfur starvation. As with the serotype transformation of paramecia and the induction and maintenance effects in *Escherichia coli*, the *Chlorella vulgaris* adaptation embodies both the characteristics of a physiological transformation and the permanency of a mutational change.

Summary

Morphological and physiological changes in populations of *Chlorella vulgaris* have been studied in order to characterize their adaptation toward the amino acid analogue selenomethionine. When grown in liquid media containing different concentrations of the analogue, approximately 50% of each population enlarged into giant forms. The total number of giants remained fairly constant throughout the entire growth curve. However, toward the end of the uncoupled growth phase when exponential divisions began, the proportion of giants that contained autospores rose markedly and then dropped to a constant value during the stationary phase, an indication that the renewed divisions of the culture had stemmed from the adapted giants. Two passages in selenomethionine sufficed to give a population marked by the absence of an uncoupled growth phase. The lag phase of the growth curve during later passages, however, consistently was slightly longer than in controls, and giants continued to be formed at a lower frequency and were smaller than in cultures exposed to the analogue for the first time.

On agar plates all cells were able to adapt and to form colonies after the usual enlargement phase. The time for colonies to first make their appearance was a function of the selenomethionine concentration. Microscopic examination revealed that adaptation of the population was heterogeneous, some cells adapting sooner than others, but out of a total of 6.5 to 7.0×10^7 cells plated thus far, none have given rise to colonies in the same time as controls. If mutants resistant to the growth uncoupling effect of selenomethionine exist they must be rare. Adapted colonies were morphologically identical with normal colonies. Subculture of their cells showed them to vary in the degree of adaptation based on the time for colonies to appear. Five serial subcultures of clones originally derived from a maximally adapted colony failed to eliminate the slight delay in their development as compared to the time taken for control colonies to appear.

All data support the view that adaptation of liquid cultures of *Chlorella vulgaris* toward selenomethionine involves a transformation of all cells rather than a selection of mutants.

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STABILITY & REVERSIBILITY OF ADAPTATION TO SELENOMETHIONINE IN CHLORELLA VULGARIS [1, 2, 3]

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Adaptation of *Chlorella vulgaris* populations to selenomethionine manifests itself as a resistance to the growth uncoupling effect of the antimetabolite. Whereas initial exposure to the antimetabolite inhibits division but allows cell growth to proceed, adapted populations will divide in the presence of the analogue without the extended period of this uncoupled growth. All cells in the population have been shown capable of the change from sensitivity to resistance (27). The present paper will show that the transformation was retained despite 220 generations in the complete absence of the amino acid analogue. Reversal to sensitivity, however, could be achieved by means of three specific environmental treatments, performed in line with the hypothesis that the adaptive mechanism involves induction of a new steady state level of the enzyme pathway leading from sulfate to methionine.

MATERIALS & METHODS

Methods for the culture and growth measurements of *Chlorella vulgaris* have been described in other papers (24, 26). The general protocol of the subcultures described in this paper is shown in table I. Inoculum sizes were kept at 2.0×10^5 cells/ml, and growth and division in most instances were allowed to continue until the deceleration or stationary phases of the growth curve had been reached. During this interval a 1,000-fold increase in cell number normally occurred, the equivalent of about ten generations.

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[3] Presented at the Pacific Slope Biochemical Conference, University of California, Davis, September 8, 1960.
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In sulfur deficient media cell number increased only about ten times or three generations. The criteria used to measure degree of adaptation were duration of the uncoupled growth phase, rate of exponential cell multiplication (calculated from the equation $k = \ln N_2 - \ln N_1/t_2 - t_1$, with t in hr), and final numbers of giant cells/ml (cells more than 10 microns in diameter). In liquid medium, cells exposed to 3.0×10^{-5} M selenomethionine for the first time characteristically show uncoupled growth that lasts approximately 150 hours; exponential cell multiplication with a calculated constant, k, of about 0.040; and a final giant count of about 1.5×10^5 cells/ml. Fully adapted cultures, on the other hand, have an uncoupled phase of about 22 hours, an average constant of 0.061, and a final giant count of about 3.0×10^4 cells/ml (*27*). Because of the variation in these features from one experiment to the next, suitable controls were run during each subculture. Points along the exponential phase of multiplication were selected and k was calculated by the method of least squares. Intercepts on the time axis of the growth curve, with the inoculum size as base line, were calculated from k and used as estimates of the durations of the uncoupled growth and lag phases.

Results & Discussion

Stability of Adaptation. Two serial subcultures of *Chlorella vulgaris* in the presence of selenomethionine will eliminate the extended phase of uncoupled growth characteristic of populations exposed to the analogue for the first time (*27*). Inasmuch as all cells can adapt, in this respect comparable to populations of microorganisms that undergo adaptive enzyme formation (*9, 13, 18, 29*), subcultures in the absence of selenomethionine were undertaken in order to determine the number of generations necessary for loss of the adaptation.

In table II are included data for a population derived from a culture grown once with selenomethionine, and once without the analogue. The cells that were then withdrawn for re-exposure to the analogue showed growth uncoupling which lasted only 87 hours. This was shorter than the 120 hours of the control, grown simultaneously, that had been exposed to selenomethionine for the first time. An increase in the frequency of divisions also occurred as seen from the exponential multiplication constant of 0.046 in contrast to 0.036 for the inhibited control. No change in magnitude of the final giant count was observed.

Rather than the expected reversal, subculture for about ten generations in the absence of selenomethionine had little or no effect on adapted cells that had been cultured once with selenomethionine. The response was similar to that obtained with these cells, during their second direct subculture in selenomethionine.

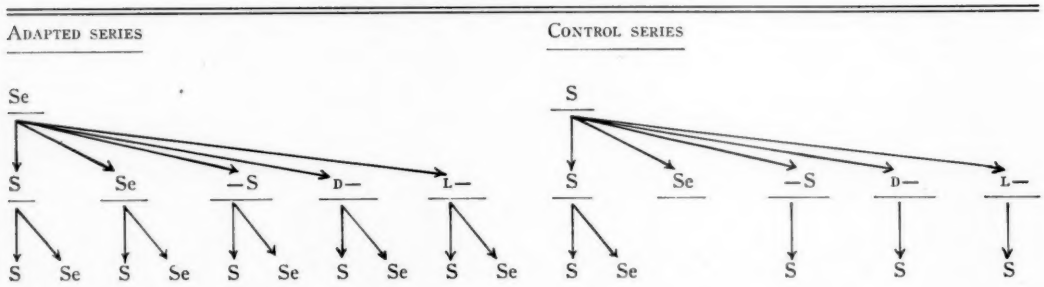
Failure to deadapt is also strikingly shown by cells drawn from a population that had first been subjected to two successive subcultures with selenomethionine (table III). After ten generations without the analogue, the descendents, on re-exposure to selenomethionine showed values of the same order of magnitude as those found in cultures directly sub-cultured with selenomethionine three times. The cells had again responded as if the intervening absence of selenomethionine had been without effect.

Identical results were obtained with cells that had been serially grown three, four, and five times with selenomethionine. Twenty generations away from the analogue had no effect on cells drawn either from cultures serially subcultured three times (table IV) or from cultures serially subcultured four times (table V) with the analogue. Resistance was also maintained by cells that had undergone five consecutive passages with the analogue, even after 30 generations without it (table VI).

A more exhaustive attempt at deadaptation was undertaken with cells that had been successively cultured six times with selenomethionine. Table VII shows that no deadaptation had occurred even after 22 passages, the equivalent of about 220 generations. The duration of the uncoupled phase during the 22

Table I

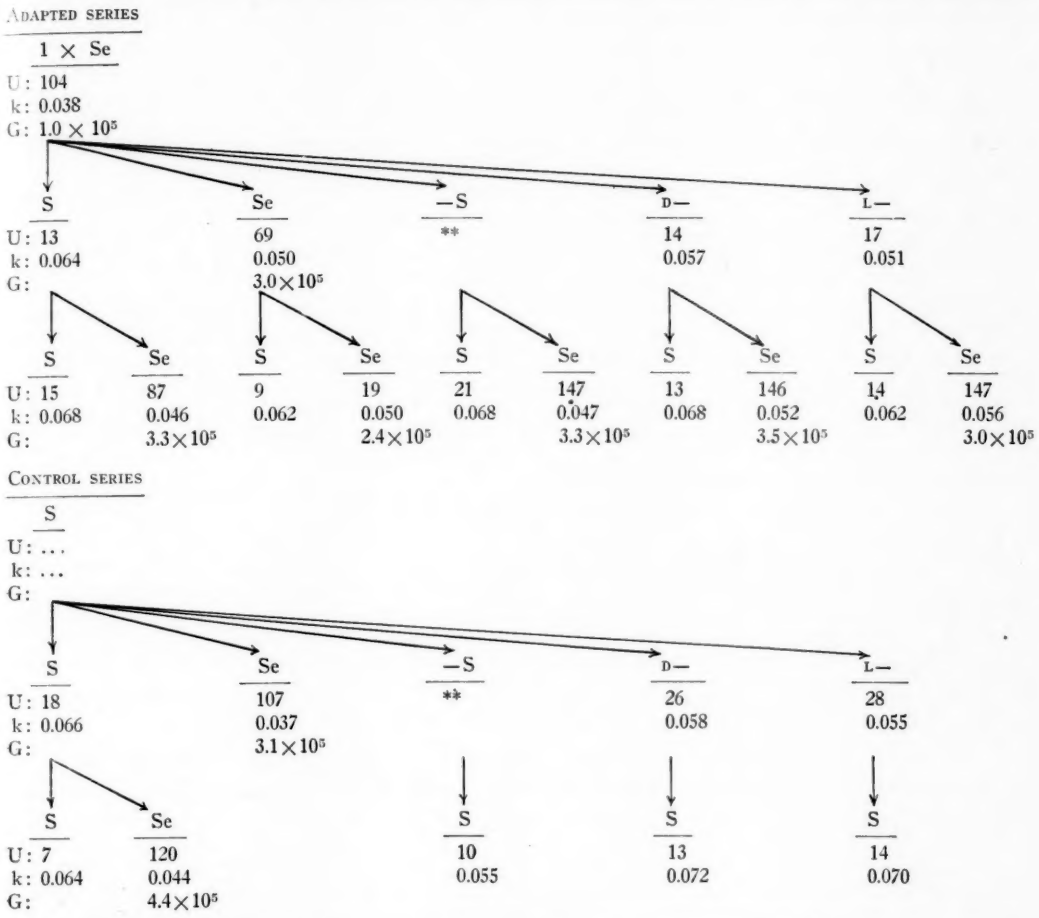
General Protocol of Subculturings



S: 3.1×10^{-4} M sulfate as only sulfur source. Se: 3.0×10^{-5} M selenomethionine; 3.1×10^{-4} M sulfate. −S: No sulfur compounds added. D−: 3.1×10^{-4} M D-methionine as only sulfur source. L−: 3.1×10^{-4} M L-methionine as only sulfur source.

TABLE II

EFFECTS OF CULTURAL CONDITIONS ON DEGREE OF ADAPTATION IN CHLORELLA VULGARIS CULTURED ONCE WITH SELENOMETHIONINE*



* U: Duration of uncoupled growth phase or of lag phase (hr). k: Exponential growth rate constant. G: Giant cells/ml at completion of subculture. Other symbols are the same as in table I.

** Final population of adapted series: 1.5×10^6 cells/ml; control series: 1.0×10^6 cells/ml.

passages ranged from 26 to 45 hours with a mean and 95% confidence interval of 33 ± 6 hours. These values are in contrast to values for cultures which on first exposure to selenomethionine exhibited uncoupled phases lasting from 130 to 165 hours. The figure 33 ± 6 is significantly larger than the mean value of 13 ± 7 obtained for the lags of the untreated control cultures that had been grown simultaneously. A comparable relationship was observed between cultures consecutively grown with selenomethionine and their untreated controls (*27*). No significant difference in exponential rates of multiplication was found between the adapted series and the untreated controls. Final numbers of giants remained unchanged.

It is evident that *C. vulgaris* populations, once they are transformed by culture in the presence of selenomethionine, are able to maintain their transformation indefinitely after removal of the causative agent. Even populations that have only partially adapted do not revert when their cells are so cultured. On the contrary, these partially adapted cells continue on their way to complete adaptation on re-exposure to selenomethionine as if the interim had been without effect. Under the conditions specified, therefore, the adaptation can be considered a stable one.

REVERSIBILITY OF ADAPTATION. The fact that the transformation is retained despite 220 generations without the analogue, probably rules out any mechanism whose explanation invokes an induction of a new enzyme system capable of either destroying or utilizing the selenomethionine. Such an adaptive enzyme would have been expected to disappear after withdrawal of the analogue, as has been established for other induced systems (9, 13, 18, 29). A testable hypothesis to explain the stable transformation can be drawn up based on the known biochemical properties of selenomethionine.

Protein hydrolysates of growth uncoupled cells lack methionine, as revealed by paper chromatography, whereas other sulfur-containing amino acids, in the chromatographic region of cyst(e)ine, are present (26). In view of the ability of selenomethionine to replace methionine in several biological systems (2, 5, 14, 17, 30) it is likely that altered proteins which contain the analogue are being synthesized by the growth uncoupled *C. vulgaris* cells. The reduction of sulfate to cysteine and the incorporation of this sulfur amino acid into protein, however, were not affected. The question arises as to whether or not the altered enzymes retain their activity. Such a retention can be inferred from the observations that large increases in dry weight, oxygen uptake, and protein nitrogen occur during uncoupled growth (26). Furthermore, a methionine-requiring mutant of *Escherichia coli* has been grown with selenomethionine in the complete absence of methionine (2, 5) and a cellular fraction, prepared from this organism, has been shown to activate selenomethionine as well if not better than methionine itself (17). There is also abundant evidence from other microorganisms that amino acid analogues can be incorporated into proteins, and that some altered enzymes nevertheless retain activity (3, 6, 8, 15, 16, 20, 34, 35).

When the growth uncoupled population resumes exponential divisions there is a return to the weight, size, and protein level of normal cells. Most significant is the fact that methionine is once more present in the proteins (26). One can therefore conclude that the adaptive process involves the development of an ability to incorporate methionine, derived from sulfate, into cellular proteins in the presence of selenomethionine.

The following assumptions have been made to explain this renewed ability:

A. The competitive biochemical block by selenomethionine, which prevents methionine entry into proteins, also causes accumulation of methionine and of intermediates on the route from sulfate to methionine. Antimetabolites have been demonstrated to cause such an accumulation of metabolites in other organisms (15, 28).

B. One might expect that the higher levels of metabolites would repress the biosynthesis of certain of the enzymes responsible for the synthesis of these metabolites, as is the case in some microorganisms (12, 19, 31). In particular, methionine has been shown to repress the formation of enzymes which lead to the synthesis of this amino acid in *E. coli* and in *Proteus morganii* (1, 4, 32, 33). If such a repression is operative in *Chlorella vulgaris*, then selenomethion-

TABLE III

FAILURE OF CHLORELLA VULGARIS, SUBCULTURED TWICE WITH SELENOMETHIONINE, TO DEADAPT AFTER SUBCULTURE WITHOUT THE ANALOGUE *

ADAPTED SERIES		CONTROL SERIES	
2 × Se		S	Se
U: 69		U: 18	107
k: 0.050		k: 0.060	0.037
G: 3.0×10^5		G:	3.1×10^5
S	Se	S	Se
U: 9	19	U: 7	120
k: 0.062	0.050	k: 0.064	0.044
G:	2.4×10^5	G:	4.4×10^5
S	Se	S	Se
U: ...	19	U: 7	140**
k: ...	0.050	k: 0.058	...**
G:	2.0×10^5	G:	1.4×10^5

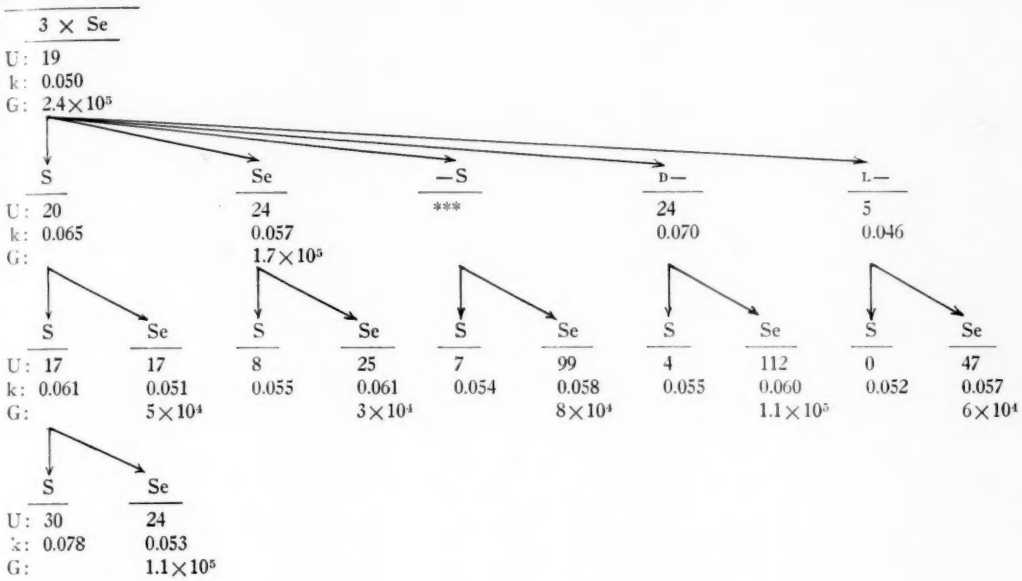
* U: Duration of uncoupled growth phase or of lag phase (hr). k: Exponential growth rate constant. G: Giant cells/ml at completion of subculture. Other symbols are the same as in table I.

** Too few points to calculate k; duration of uncoupled phase estimated.

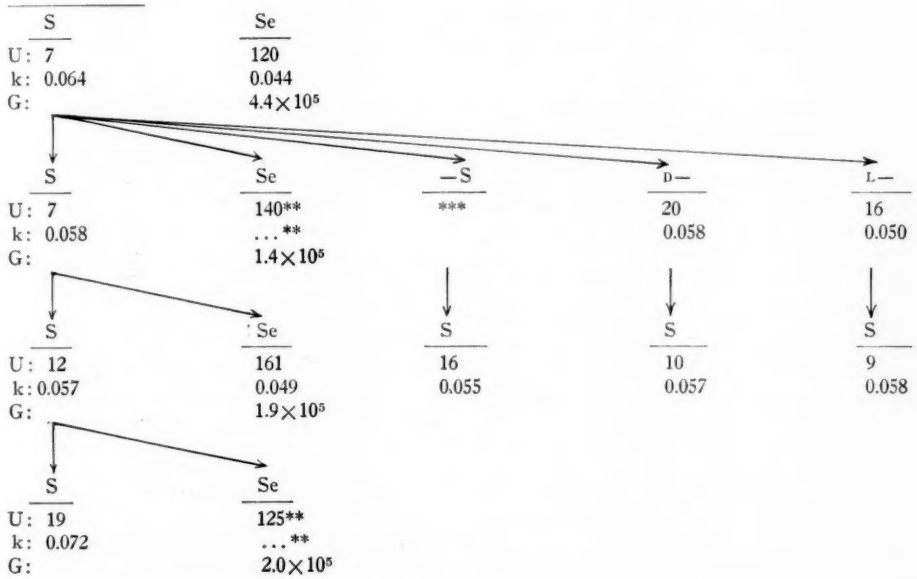
TABLE IV

EFFECTS OF CULTURAL CONDITIONS ON DEGREE OF ADAPTATION IN CHLORELLA VULGARIS AFTER THREE SERIAL SUBCULTURES WITH SELENOMETHIONINE *

ADAPTED SERIES



CONTROL SERIES



* U: Duration of uncoupled growth phase or of lag phase (hr). k: Exponential growth phase constant. G: Giant cells/ml at completion of subculture. Other symbols are the same as in table I.

** Too few points to calculate k; duration of uncoupled growth phase estimated.

*** Final population of adapted series: 3.2×10^6 cells/ml; control series: 2.8×10^6 cells/ml.

TABLE V

FAILURE OF CHLORELLA VULGARIS, SUBCULTURED 4 TIMES WITH SELENOMETHIONINE, TO DEADAPT AFTER SUBCULTURE WITHOUT THE ANALOGUE*

ADAPTED SERIES		CONTROL SERIES	
4 × Se		S	Se
U: 24		U: 7	140**
k: 0.057		k: 0.058	...**
G: 1.7×10^5		G:	1.4×10^5
S	Se	S	Se
U: 8	25	U: 12	161
k: 0.055	0.061	k: 0.057	0.049
G:	3×10^4	G:	1.9×10^5
S	Se	S	Se
U: 29	32	U: 19	125**
k: 0.077	0.067	k: 0.072	...**
G:	1.2×10^5	G:	2.0×10^5
S	Se	S	Se
U: 21	25	U: 19	134
k: 0.078	0.069	k: 0.065	0.031
G:	4×10^4	G:	2.1×10^5

* U: Duration of uncoupled growth phase or of lag phase (hr). k: Exponential growth rate constant. G: Giant cells/ml at completion of subculture. Other symbols are the same as in table I.

** Too few points to calculate k; duration of uncoupled growth phase estimated.

ine, or the accumulated, endogenously produced methionine, or both could be expected to enhance the repression during the period of uncoupled growth. This might account for the failure to detect methionine in the proteins of the uncoupled cell, but it would not adequately explain either the reappearance of methionine in the proteins when exponential divisions resume or the maintenance of the adaptation after removal of the analogue.

There is evidence that certain constitutive enzymes can be induced to higher levels if their substrates are exogenously supplied to the cells (11, 22). In another instance, a temporary increase in constitutive enzymes, induced by a product of an enzyme sequence, has been noted (23). On this basis, one can reason that the higher levels of sulfur intermediates within *C. vulgaris* will induce an increased synthesis of one or more of the constitutive enzymes responsible for the further metabolism of these intermediates.

C. The enzyme pathway, now operating at a new steady state level, will lead to a more rapid rate of methionine synthesis, the consequent displacement of the selenomethionine, a reappearance of methionine in the cellular proteins, and a renewed ability of the uncoupled cell to divide at exponential rates.

D. As long as the adapted cells are given sulfate, even in the absence of selenomethionine, the high rate of methionine synthesis should be maintained indefinitely, for according to this picture the actual inducer of the new enzyme level is not selenomethionine but one or more of the endogenous sulfur intermediates.

Three types of experiments have been performed, based on these assumptions, in an effort to cause reversion of the adapted cells to their original state of sensitivity toward selenomethionine. The rationale for these experiments has been as follows:

A. SULFUR STARVATION: this treatment should lead to a depletion of endogenous sulfur intermediates and a consequent lowering of enzyme levels during the limited population increase, amounting to two or three generations, that occurs under such resting state conditions. The starved cells, on re-exposure to selenomethionine, should once more be sensitive to the analogue.

B. CULTURE WITH D-METHIONINE: since *C. vulgaris* is able to use D-methionine as its only source of sulfur (24, 25), the route from sulfate to methionine can be circumvented. Growth of adapted cells with this isomer should therefore also lead to de-

TABLE VI

EFFECTS OF CULTURAL CONDITIONS ON DEGREE OF ADAPTATION IN CHLORELLA VULGARIS AFTER 5 SERIAL SUBCULTURES WITH SELENOMETHIONINE*

ADAPTED SERIES

	5 × Se
U:	25
k:	0.061
G:	3.0×10^4

	S	Se	−S	D−	L−
U:	21	27	***	28	19
k:	0.076	0.066		0.075	0.058
G:		1.0×10^5			

	S	Se	S	Se	S	Se	S	Se	S	Se
U:	22	28	23	21	23	81	20	104	21	57
k:	0.081	0.075	0.084	0.066	0.069	0.054	0.079	0.067	0.083	0.066
G:		5×10^4		5×10^4		5×10^4		6×10^4		6×10^4

	S	Se
U:	14	17
k:	0.068	0.063
G:		3×10^4

	S	Se
U:	29	32
k:	0.073	0.064
G:		4×10^4

CONTROL SERIES

	S	Se
U:	12	161
k:	0.057	0.049
G:		1.9×10^5

	S	Se	−S	D−	L−
U:	19	125**	***	26	20
k:	0.072	...**		0.069	0.055
G:		2.0×10^5			

	S	Se	S	S	S
U:	19	134	30	23	20
k:	0.065	0.031	0.076	0.074	0.079
G:		2.1×10^5			

	S	Se
U:	7	130**
k:	0.061	...**
G:		2.3×10^5

	S	Se
U:	14	165**
k:	0.064	...**
G:		1.3×10^5

* U: Duration of uncoupled growth phase or of lag phase (hr). k: Exponential growth rate constant. G: Giant cells/ml at completion of subculture. Other symbols are the same as in table I.

** Too few points to calculate k; duration of uncoupled growth phase estimated.

*** Final population of adapted series: 1.5×10^6 cells/ml; control series: 2.3×10^6 cells/ml.

pression of intermediates and their respective enzymes.

C. Culture With L-Methionine: L-methionine also serves as an only sulfur source (24, 25); the same argument could be expected to hold here.

Table II, discussed previously in connection with the failure of cells to deadapt after ten generations away from the analogue, summarizes the effects of the reversal conditions on cells that previously had been cultured once in the presence of selenomethionine. Unlike the subculture in complete medium, with sulfate as sulfur source, each of the three treatments resulted in deadapted cells. After sulfur starvation, re-exposure to selenomethionine gave an uncoupled phase that lasted 147 hours and a growth rate constant of 0.047. The selenomethionine control, grown simultaneously, gave comparable values of 120 hours and 0.044. Treatment with D-methionine gave rise to cells whose uncoupled phase lasted 146 hours, but which divided at a somewhat faster rate, the growth rate constant being 0.052. After L-methionine treatment, the uncoupled phase also lasted 147 hours, but the growth rate constant was 0.056. In all three cases the final number of giants was of the same order of magnitude as in the selenomethionine control. It should be noted that under conditions of sulfur starvation in this experiment, the population multiplied from 0.22×10^6 cells/ml to only 1.5×10^6 cells/ml, an increase of approximately three generations. The subsequent growth pattern in the presence of selenomethionine, therefore, cannot be attributed to selection of sensitive mutants during the starvation period. In the two methionines, about nine generations developed between the time of inoculation and withdrawal of inoculum cells for re-exposure to selenomethionine.

That the three treatments in themselves had no adverse effects can be seen from the growth characteristics of the so-treated controls and adapted cells that were transferred to complete medium. Even sulfur-starved cells grew normally. Enlarged cells typical of another species, *Chlorella ellipsoidea*, during sulfur starvation (10) were not observed.

A second deadaptation trial in which D-methionine culture and sulfur starvation were applied to adapted cells that had been grown once with the analogue gave identical results.

Cells grown three times with the analogue were also subjected to the three conditions (table IV). In contrast to the selenomethionine control which had an uncoupled growth phase of 161 hours and a division rate constant of 0.049, sulfur starvation gave rise to cells whose uncoupled growth lasted 99 hours and whose exponential divisions had a constant of 0.058; D-methionine treatment caused an uncoupled period of 112 hours and a constant of 0.060; and L-methionine subculture gave cells with an uncoupled phase of 47 hours and a constant of 0.057. None of the treatments caused an increase in final giant/ml count to the level of the selenomethionine control, except perhaps the D-methionine subculture. Deadaptation in terms of the criteria used therefore appears to have been less complete with these adapted cells than with adapted cells grown once with the analogue.

Table VI shows that incomplete deadaptation could also be brought about in cells that had been serially subcultured five times with selenomethionine.

Similar data were obtained with cells that had first been subcultured six times with selenomethionine followed by 13 passages without it (table VIII, fig 1). Whereas the 13 passages had had no effect on the degree of adaptation, each of the three treatments

Table VII

Failure of Chlorella Vulgaris, Subcultured 6 Times With Selenomethionine, to Deadapt After 22 Subcultures Without the Analogue

Adapted series				Control series				
				+ Selenomethionine			Untreated	
No. passages without selenomethionine	Uncoupled phase after transfer to selenomethionine (hr)	Slope of exponential phase	Giants at end of subculture 10^5 cells/ml	Duration of uncoupled phase (hr)	Slope of exponential phase	Giants at end of subculture 10^5 cells/ml	Duration of lag phase (hr)	Slope of exponential phase
1	29	0.069	0.3	130*	...*	2.3	7	0.061
2	45	0.071	0.1	165*	...*	1.3	14	0.064
13	26	0.077	0.3	160*	...*	2.0	5	0.063
14	27	0.075	0.3	162	0.044	1.8	6	0.068
15	39	0.053	0.1	150	0.043	2.4	22	0.073
17	36	0.055	0.2	145*	...*	1.3	23	0.072
22	32	0.069	0.1	...	...	...	14	0.059
	33 ±6**	0.067 ±0.009**					13 ±7**	0.066 ±0.001**

* Too few points to calculate k; duration of uncoupled growth phase estimated.
** Mean & 95 % confidence interval

TABLE VIII

EFFECTS OF CULTURAL CONDITIONS ON DEGREE OF ADAPTATION IN CHLORELLA VULGARIS AFTER 6 SERIAL SUBCULTURES WITH, & 13 SUBCULTURES WITHOUT, SELENOMETHIONINE*

ADAPTED SERIES

6 × Se → 13 × S

	S	Se	−S	D−	L−
U:	18	26	***	6	12
k:	0.078	0.077		0.054	0.054
G:		3×10⁴			

	S	Se	S	Se	−S	S	Se	D−	S	Se	L−
U:	16	27	16	108	†	...	89	10	10	59	2
k:	0.074	0.075	0.079	0.065		...	0.064	0.056	0.071	0.073	0.045
G:		3×10⁴		5×10⁴			3×10⁴			4×10⁴	

	S	Se	S	Se	S	Se	S	Se
U:	24	39	18	131	23	118	27	91
k:	0.068	0.053	0.058	0.037	0.066	0.043	0.073	0.064
G:		1×10⁴		3×10⁴		1×10⁴		3×10⁴

CONTROL SERIES

S

	S	Se	−S	D−	L−
U:	5	160**	***	12	15
k:	0.063	...**		0.056	0.056
G:		2×10⁵			

	S	Se	S	−S	S	D−	S	L−
U:	6	162	16	†	14	13	6	...
k:	0.068	0.044	0.065		0.079	0.061	0.064	...
G:		1.8×10⁵						

	S	Se
U:	22	150
k:	0.073	0.043
G:		2.4×10⁵

* U: Duration of uncoupled growth phase or of lag phase (hr). k: Exponential growth rate constant. G: Giant cells/ml at completion of subculture. Other symbols are the same as in table I.

** Too few points to calculate k; duration of uncoupled growth phase estimated.

*** Final populations of adapted series: 3.2×10^6 cells/ml; control series: 4.7×10^6 cells/ml.

† Final population of adapted series: 3.0×10^6 cells/ml; control series: 2.7×10^6 cells/ml.

lengthened the uncoupled growth period. Depression of the growth rates was not as marked, however, nor were there significant increases in the final numbers of giants/ml. An additional subculture of each type caused further increases in the uncoupled phases, and except for the L-methionine treatment, further declines in the division rates. Final numbers of giants remained unaffected. After 220 generations without the analogue, descendents of these adapted cells also deadapted with these treatments and to the same extent observed after 13 subcultures without the analogue. The selenomethionine control, in this experiment, had an uncoupled phase that lasted, unaccountably, for 202 hours.

All the physiological reversal experiments have proven successful. Populations exposed once to selenomethionine deadapted completely. Least deadaptation occurred with cells that had been serially subcultured several times with selenomethionine. Here the inability to completely revert was most apparent from the failure of giant cells to develop with the frequency found in cultures exposed to selenomethionine for the first time. Whereas first exposure to the analogue inhibits the division mechanism and allows cell growth to continue before exponential divisions resume, deadaptation of fully adapted cells yielded populations whose division was inhibited, and growth as well, to judge from the low frequency of giants. One possible explanation is that the duration of the sulfur starvation treatment and the number of generations with D- or L-methionine were insufficient. The least effective of the deadaptation procedures was consistently the subculture with L-methionine. The reasons for this undoubtedly lie in the unknown metabolism of this isomer. The biochemical pathways whereby the sulfur of both isomers of methionine is transformed into all other sulfur metabolites required for normal growth of the alga is completely unknown.

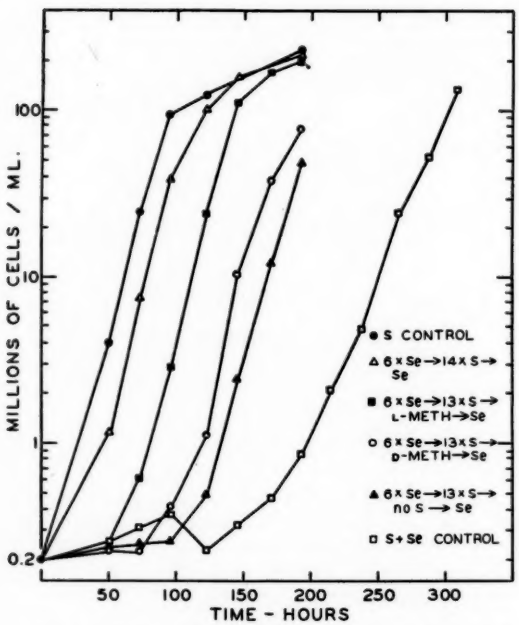


FIG. 1. Effects of cultural conditions on degree of adaptation in *Chlorella vulgaris* after 6 serial subcultures with, and 13 subcultures without selenomethionine. See table VIII for complete data.

Despite the obscurity of the intermediate steps in the reduction of sulfate and utilization of methionine sulfur in *C. vulgaris*, the successful deadaptation attempts in all trials lend validity to the assumptions made earlier in explanation of the adaptive mechanism. It thus appears possible to induce a population of cells to develop a new steady state level of constitutive enzymes, and to have this transformation passed on to the progeny indefinitely after complete removal of the causative agent. Theoretical treatments of such transformations have been discussed by Delbruck (7) and by Pollock (21).

SUMMARY

Resistance to the growth uncoupling effect of selenomethionine, an adaptation which develops in all cells of *Chlorella vulgaris*, was maintained despite removal of the analogue. Cells that had undergone from one to six consecutive subcultures with the antimetabolite were subcultured without it and on reexposure were as resistant as before. As many as 22 passages away from selenomethionine, the equivalent of 220 generations, failed to cause deadaptation of cells derived from a population that had previously been cultured six times with the analogue. Complete or partial reversal could be achieved at any time, however, if adapted cells were subjected to sulfur starvation, subculture with D-methionine as only sulfur source, or subculture with L-methionine as only sulfur source. The reversal data fit the hypothesis that the permanent adaptation to selenomethionine in this alga involves induction of a higher steady state level of the enzymes responsible for reduction of sulfate to methionine.

ACKNOWLEDGMENTS

The authors wish to thank Professor John R. Preer and Mrs. Lolita Moore for many fruitful discussions.

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NEWS & NOTES

NORTHEASTERN SECTION MEETING, AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS. The Annual Meeting of the Northeastern Section was held May 12 and 13, under the chairmanship of Dr. Stuart Dunn in the new Spaulding Life Science Building, University of New Hampshire. More than 100 members, representing all 10 northeastern states, were welcomed by Dr. John F. Reed, ecologist, dean of the Graduate School and of the College of Liberal Arts. Friday afternoon and Saturday morning 30 research reports were presented. The after-dinner address Friday night was a most interesting thesis about the role of vegetation in the genesis of "natural" smogs, "Why The Mountains Look Blue", by Dr. Fritz Went, director, the Missouri Botanical Garden.

The Section was invited by Dr. W. Rei Robbins, chairman of the faculty of botany, Rutgers, to meet in the spring of 1962 at New Brunswick. Officers elected for 1962 are: Dr. Harold E. Clark, chairman; Dr. Jerome A. Schiff, vice chairman, and Drs. Martin Gibbs, Carl A. Price, and Milton Zucker, new members of the executive committee. Although by vote of the attending membership dues were left unchanged, everyone attending the Annual Section Meeting will be charged an additional 75c to help defray meeting expenses.

—HUBERT J. DYER, *Secretary-Treasurer,*
Northeastern Section.

SYMBOLS & ABBREVIATIONS

COMMONLY USED IN PLANT PHYSIOLOGY

General Rules for Abbreviations

Spell Out

- Words in title & summary (except common chemical symbols & exceptions noted below)
- Words & numerals which begin a sentence
- Units of time (except in tables & parentheses): 30 mg, *but* 50 seconds

Abbreviate

- In parentheses & tables (where possible): (fig 4), (dry wt)
- Units of measurement in text (only when preceded by numeral)

Symbols & Abbreviations*

A-B	about (*circa*)	ca.
	absolute	abs
	absorbancy	*A*
	adenosine diphosphate	ADP
	adenosine triphosphate	ATP
	and	& (in parentheses, tables, titles)
	and others (*et alii*)	et al.
	Ångstrom unit(s) (10^{-8} cm)	A (*not* Å)
	approximate	approx (in tables)
	aqueous	aq
	atmosphere(s)	atm
	average	avg (in tables)

(*Continued on next page*)

* Abbreviations (with a few exceptions) follow the "Style Manual for Biological Journals," 1960, American Institute of Biological Sciences (Library of Congress Catalog No. 60-15133).

SYMBOLS & ABBREVIATIONS

Continued from page 521

C	centimeter(s)	cm
	coenzyme A (& its acyl derivatives)	CoA
	concentration	conc (in tables)
	configuration	D-, L-, DL-, [*but d-, l-, dl-* or (+)- & (−)-]
	counts per minute	cpm
	cubic centimeter(s)	cc (or cm^3)
	curie(s)	c
D-E	degree(s), centigrade	C (omit degree symbol)
	deoxyribonucleic acid	DNA
	diameter	diam (in tables)
	diphosphopyridine nucleotide	DPN
	disintegrations per second	dps
	dry weight	dry wt (in tables)
F	figure	fig (in parentheses & tables)
	-fold	threefold, manifold, *but* 12-fold
	foot candle(s)	ft-c
	fresh weight	fr wt (in tables)
G	gram(s)	g
	gravity	*g*
H	hour(s)	hr (in tables & parentheses)
I-J	indoleacetic acid	IAA
K	kilogram(s)	kg
L	least significant difference	LSD
	liter(s)	l (only in tabular matter because the abbreviation is easily confused with the figure "1")
M	maximum, maximal	max (in tables)
	meter	m
	microgram(s)	μg
	microliter(s)	μl
	micromolar(s) (unit of conc)	μM
	micromole(s) (unit of mass)	μmole
	milliequivalent(s)	meq
	milligram(s)	mg
	milliliter(s)	ml

(*Continued on next page*)

Continued from page 522

M cont. ...	millimicron(s)	mμ
	millimolar (unit of conc)	mM
	millimole(s) (unit of mass)	mmole
	minimum, minimal	min (in tables)
	minute(s)	min (in tables & parentheses only)
	molar (mole/liter)	M
	mole(s) (a gram molecule)	mole
N-O	normal (conc)	N
	normal (in trivial names in organic compounds)	*n*-
	number	No.
P	page	p (plural—pp)
	part(s) per million	ppm (*not* p. p. m.)
	per cent	% (with numeral & in tables)
	precipitate	ppt (in tables)
R	revolutions per minute	rpm
	ribonucleic acid	RNA
S	second(s)	sec (in tables only)
	species	sp. (when part of bionomial)
	square centimeter(s)	cm^2
	standard deviation	SD
	standard error	SE
T-U	temperature	temp (in tables)
	time measurements	(abbreviate in tables & parentheses only)
	triphosphopyridine nucleotide	TPN
V	variety(ies) (in taxonomy)	var. (when used with the binomial)
	versus	vs.
	volt(s)	v
	volume(s)	vol (in tables)
	volume/volume (conc)	v/v
W-Z	watt(s)	w
	weight	wt
	weight/volume (conc)	w/v

INSTRUCTIONS FOR CONTRIBUTORS TO PLANT PHYSIOLOGY

I. Writing Manuscript.

A. Style. In general, the style of an article will reflect the author's preference and habits of writing. The Editor may suggest improvements, but will not insist on rigid conformity to conventions at the expense of an author's individuality. Scientific writing, however, should meet accepted standards of grammar, syntax, and diction.

Strive for specific, concise expression. Use active verbs. Avoid complicated sentence structure. Write in a straightforward manner. Prepare the original manuscript carefully to obviate subsequent delays in publication because of the need for manuscript revision.

B. Length. Beyond ten pages (including tables, figures, & literature citations) authors will be billed $20 per page for overrun. Approximately four double spaced typewritten pages (pica type) equal one page of printed type.

C. Footnote References. These should be avoided in the body of the text, but if a footnote must be used, it should be numbered with an Arabic numeral and typed at the bottom of the page on which reference to the footnote is made.

D. Acknowledgments. Do not include acknowledgments in a footnote; they belong under their own heading following the SUMMARY and preceding LITERATURE CITED.

E. Punctuation.

1. Periods and Commas. These are the most often used and misused punctuation marks; the period is not used enough, the comma too often. Avoid complicated sentences which require many commas, colons, or semicolons. Break such long sentences into brief, concise sentences which will be more easily understood.

2. Quotation Marks. Avoid the urge to use excessive quotation marks. Often a word is used in a sense slightly different from its ordinary meaning. This does not necessarily warrant the use of quotation marks. In case a term is used in an unconventional way and the ambiguity might lead to misunderstanding, use quotation marks only when the unfamiliar meaning is first explained.

3. Brackets. Material is customarily enclosed in single brackets: ( ). Where double brackets are necessary use the following: [( )]. Abbreviations are the rule: &, fig, hr, min, sec.

4. Hyphens. Use sparingly.

F. Subtopics. Use this order and style to number subtopics:

I.	I)
A.	A)
1.	1)
a.	a)

G. Scientific Names. The complete botanical name (genus, species, & authority for the binomial) of all experimental plants should be included, under MATERIALS AND METHODS. Refer to the most recent International Code of Botanical Nomenclature for rules governing the use of scientific names.

In general PLANT PHYSIOLOGY follows the style recommenations of the Conference of Biological Editors—American Institute of Biological Sciences, Committee on Form and Style. 1960. Style manual for biological journals. Am. Inst. Biol. Sci., Washington.

H. Abbreviations.

In place of certain unwieldy chemical names abbreviations may be used as a convenience. The writing, however, must remain readily comprehensible and unambigious. For the most part only widely used abbreviations should be employed (e.g.: ATP, RNA,). Standard chemical symbols may be used without definition: Ca, NaOH. All others should be defined when they first appear. Example: The reaction was thought to involve flavin adenine dinucleotide (FAD). If your article uses several abbreviated forms it is permissible to define them all in a single footnote where the first abbreviation is introduced.

In no case should the number of nonstandard abbreviations be so large that the reader is forced to learn a special vocabulary in order to read your article easily. Abbreviations should not frustrate the reader; they should be used for his convenience, not the author's convenience.

In title and summary avoid abbreviations which are not generally accepted. Because titles and summaries frequently are translated into foreign languages, undefined abbreviations may be confusing.

I. Chemical Formulae. It is expensive (sometimes impossible) to set formulae in type. To avoid errors, submit structural formulas or involved reaction sequences as clearly executed India ink drawings which can be photographed. Indicate where these are to be inserted into the

text. Alternatively a complex reaction diagram or flow sheet may be treated as a regular figure, given a number, and supplied with a legend.

II. PREPARING TYPESCRIPT.

Use bond paper for the first copy in preference to onion skin. Consult recent editions of PLANT PHYSIOLOGY for proper placement of main headings, paragraph headings, and subheadings.

A. SPACING. Double space EVERYTHING, including tables, legends, literature cited, and footnotes. It is essential that all copy be double spaced to allow room for the extensive proofmarking which must be done (even to a perfect typescript) in the editorial office so the printer will know precisely how to set your copy in type.

Begin typing 1/3 of the way down the first page; leave liberal side margins.

Double space each table, each set of figures, each set of legends, and each set of literature citations on a separate page; these items are given by the printer to different people for setting in type sizes different from that in the body of the manuscript.

B. ORDER. Arrange manuscript copy in this order:

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3. Author's name and institution
4. Text
5. Summary
6. Acknowledgments
7. Literature cited
8. Tables
9. Legends for figures
10. Figures

C. PAGING. Number all pages consecutively (including tables & legends) in upper right hand corner. Never use a letter such as 2a, 5D, for paging; such pages are easily lost during processing.

D. PAGE ONE. Do not use a title page.

1. *Name and Address.* On page 1 above the title of the article, put the name and address of the person to whom editorial correspondence (including galley proof & reprint order form) is to be sent. The name and address of the institution where the work was done follow the name of the author directly below the title.
2. *Footnotes.* One or more footnotes are included at the bottom of the first page. The first footnote (indicated by superscript 1 after the title) gives the date the manuscript was received. The second footnote credits the source of financial support (if any). Additional footnotes give the present address of the author, if different from the address in the heading, and indicate the experiment station or institution paper number. Normally these are the only footnotes used.

E. NUMBERS.

1. Write out all numbers (cardinal & ordinal) from one through ten. Use figures for all numbers larger than ten. Exceptions:
   a. When using precise measurements such as 5 per cent, 6 mm, 8-hour cycle, figure 1. In nonprecise usages spell numbers out: about two weeks, six or eight degrees.
   b. In a series of related numerical expressions treat all alike even though violating the general rule: 2 leaves, 8 leaves, and 12 leaves.
   c. When a single expression has consecutive numbers, use both figures and spelled out numerals: two 6-minute exposures.
   d. Spell out ALL numbers or fractions which begin a sentence. If this is awkward, rephrase the sentence to avoid beginning with a numeral.
   e. Do not use a hyphen to replace the preposition "to" between numerals: 13 to 22 minutes, 3 to 10 °. Exceptions: tables, figures, graphs.

F. FRACTIONS. Write out and hyphenate nonprecise simple fractions: two-thirds, *but* half strength. Exceptions: tables, figures, graphs, legends, fractions in parentheses. Often it is desirable to use decimals instead of fractions. For values less than one, insert a zero before the decimal point: 0.7 g *not* .7 g.

G. LITERATURE CITED. This section of a manuscript requires extensive proofmarking even though copy is correct in every detail. Typing errors are easily made, but difficult to detect, so exercise special care in preparing and checking the bibliography. Since they are set in a typeform different from that used for the body of the manuscript, literature citations should be *double spaced on a separate piece of paper.* Consult the most recent issue of PLANT PHYSIOLOGY for Journal style. Note the limited use of capitals.

Check all citations (such as title, volume number, pages, & date) for completeness. Check foreign titles for diaresis and other markings. Make certain that all citations are referred to in the text.

List citations in alphabetical order (not in order of reference), then number them and indicate citations in the body of the text by Arabic numerals enclosed in parenthesis.

Unpublished data, personal communications, and articles in preparation are not acceptable as literature citations, but should be referred to parenthetically in the text. Articles that are in press may be so designated in LITERATURE CITED. An article is not properly referred to as "in press" unless it has been *accepted* for publication. The journal in which an in press article will appear should be included in the literature citation.

1. Authorship. List family name of first or sole author before listing initials or given name. Thereafter write names and initials (or given name) of all coauthors in their natural order. Use initials for a man's given name; write out a woman's given name: Reeves, A. C., R. D. Jones, and Helen Smith.

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III. PREPARING TABLES.

Set up tables in a form consistent with recent issues of PLANT PHYSIOLOGY. Number tables consecutively in Roman numerals and place the author's name on the back of each page.

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On the average, about 52 typewriter spaces (excluding the new executive typewriter) fill one 3-inch line of type of the size normally used in tables; ten double spaced typewritten lines require 1 inch of the available vertical printed space.

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C. FOOTNOTE DESIGNATIONS. Refer to footnotes by the asterisk, double asterisk, triple asterisk, dagger, double dagger, and section, in this sequence as needed: *, **, ***, †, ‡, §. *Place all explanatory material in footnotes* rather than in the heading or columnar headings, both of which should be as *brief as possible* without sacrificing clarity.

D. HEADINGS & FOOTNOTES. Wherever possible, omit unnecessary articles such as "the", "a", in headings and notes. Also avoid bulky prepositional phrases.

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A. PRINTS FOR REVIEW. For manuscript review it is satisfactory to furnish illustrations which have been reproduced by such economical copy methods as Verifax and blue print. Thus prints which will be used for publication need not be sent through the mail repeatedly. *Do not send original drawings or photos* at any time.

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Send the original copy of the manuscript, the first carbon, and two copies of all illustrative material to the Editor, Dr. Allan H. Brown; Department of Botany; University of Minnesota; Minneapolis 14, Minn. Retain a carbon copy of your manuscript and original photographs or drawings to insure against loss.

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VI. Processing Manuscript.

A. Review. The manuscript usually will be sent to two reviewers familiar with that field of research covered by the paper. Each reviewer evaluates the manuscript, suggests improvements, and recommends accepting or rejecting the paper. Usually two months suffice for review and decision. When there is marked disagreement between reviewers, the Editor may appoint a referee (often a member of the Editorial Board) to evaluate the manuscript in light of the reviews; the referee recommends either for or against acceptance.

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Retype each page on which there are extensive changes.

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AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

Organized in 1924 for the advancement of Plant Physiology as a pure and applied field of botanical science

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